STUDIES ON *IN VITRO* PRODUCTION OF SECONDARY METABOLITES FROM MEDICINAL PLANTS

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CERTIFICATE

This is to certify that the work presented in the thesis entitled "Studies on in vitro production of secondary metabolites from medicinal plants" is based on the original research done by Mrs.K.S.Shylaraj, under my guidance and supervision, at the Department of Biotechnology and no part of the work has been included in any other thesis for the award of any degree.

M. Chandrasekaran.

DECLARATION

I hereby declare that the work presented in this thesis is based on the original work done by me under the guidance of Dr.M.Chandrasekaran, Professor and Head, Department of Biotechnology, Cochin University of Science and Technology, and that no part of this thesis has been included in any other thesis submitted previously for the award of any degree.

K.S.Shylaraj

Kochi 682022

27th August 1998

Dedicated to

My Mother,

Husband & Children

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Shylaraj. K.S.

List of abbreviations

Eph	Ephedrine
Peph'	Pseudoephedrine
2,4-D	2,4-Dichlorophenoxy acetic acid
NAA	α-Naphthalene acetic acid
IAA	Indole-3-acetic acid
BAP	6-Benzyl amino purine
Kinetin	6-Furfuryl aminopurine
IBA	Indole butyric acid
SH	Schenk and Hildebrandt
N&N	Nitsch and Nitsch
B5	Gamborg's B_5
MS	Murashige and Skoog
EMS	Ethyl Methane Sulphonate
AO	Acridine orange
UV	Ultraviolet
DMSO	Dimethyl sulphoxide

CONTENTS

CHAPTER 1

Introduction

1.1 Preface

The secondary metabolites are a complex array of organic chemicals synthesized by plants that do not seem to play any direct role in their growth and development. They are mainly produced as a defence against herbivores, microorganisms and ecological variations (Wink, 1988). More than 50,000 of these chemicals have been reported which are all different and have a restricted distribution in the plant kingdom. Many of these are ecologically and physiologically significant effectors for interaction between plant and other organisms. The secondary metabolites have a broad range of application in the pharmaceutical, chemical and food industry (Barz & Ellis, 1987; Fowler, 1987; Furuya, 1988; Fowler & Stafford, 1992). Although classified as "metabolic by-products", secondary metabolites have been described as products formed from endogenous compounds undergoing synthesis, metabolism and catabolism by the action of specialized proteins (Luckner & Nover, 1977). The production of secondary metabolites are triggered by the processes of cell differentiation (Yeoman *et al.* 1982) or represent an aspect of process of plant development (Endress, 1994). The secondary metabolites are characterized by extreme chemical diversity, with an array of simple amines and compounds ranging from esters of molecular weight less than 100 to complex polynuclear heterocyclic compounds and conjugates with molecular weight in excess of 1000 (George & Ravishankar, 1996).

The plant kingdom remains as an excellent source for phytochemicals, including pharmaceuticals, agrochemicals, flavours, fragrances and colours (Balandrin *et al.*, 1985). Unfortunately, of the estimated total of 2,50,000 to 5,00,000 existing species of higher plants, only 5-15% have been examined for their possible medicinal application (Fransworth & Binjel 1977; Spjut 1985).

In principle, it should be possible to produce any compound found in the parent plant by exploiting the chemical totipotency of plant cell. Routier and Nickell (1956) first discussed the use of plant cell culture technique for commercial production of natural products. Since then a wide range of compounds viz. alkaloids, steroids, terpenes, flavonoids etc. have been obtained by this technique (Phillipson, 1990). Further, it has been shown that the production potentials of cultured cells are equal or often higher than the parent plant (Staba, 1985). Hence, plant cell cultures offer a potential alternative for phytochemicals which are of importance to food and pharmaceutical industries (Fowler & Stafford, 1992). A few products that have been developed on industrial level by using cell culture technology are listed in Table 1.1.

The production of phytochemicals *in vitro* is advantageous in many ways (Chrispeels & Sadava, 1994): (a) It is independent from various environmental factors such as climate, diseases, geographical and seasonal constraints, (b) it is a defined production system with production, as and when required, giving a close control over market supply, (c) the product quality and yield are more constant, (d) it is

Table 1.1

Plant tissue cultures developed for industrial application*

Product	Species	Company	Country
Shikonin	*Lithospermum erythrorhizon*	Mitsui	Japan
Berberine	*Coptis japonica*	Mitsui	Japan
Biomass	*Panax ginseng*	Nitto Denki	Japan
Peroxidase	*Raphanus*	Toyobo	Japan
Geraniol	*Geranium*	Kanebo	Japan
Rosmarinic acid	*Coleus blumei*	Natterman	Germany
Digoxin	*Digitalis lanata*	Boehringer Mannheim	Germany

*Crispeels & Sadava (1994).

independent of political interference, (e) it is possible to produce novel compounds of commercial interest and (f) it is easy to manipulate.

Most of the plant derived toxins have found medicinal application as drug entities or as model compounds or templates for drug synthesis and semisynthesis (Fransworth & Soejarto, 1985). Most of these metabolites have highly complex stereostructures with many chiral centres which may be essential for biological activity and hence cannot be synthesized economically on a commercial basis (Nakanishi, 1982). The increasing consumer demand for natural products rather than synthetic products encourages the use of plant extracts. Land scarcity is a serious problem for the large scale cultivation of medicinal plants. Further, the supply, cost and quality of raw materials are all affected by climate, diseases and by the political instability in the producing countries. All these problems can be avoided by cost effective plant cell cultures.

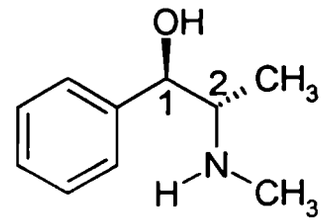
The use of medicinal plants for the relief of several diseases is an art as old as mankind. Medicinal plant research had itsups and downs during the last decade in many industrialised countries, but substances derived from higher plants constitute nearly 25% of the prescribed medicines (Paul *et al.* 1992). The alkaloids are the most familiar class of plant toxins of which more than 5,000 different ones have been described. As they affect the central nervous system, they are widely used in medicine (for example morphine, codeine, atropine and ephedrine) and also as stimulants or sedatives (caffeine, cocaine and nicotine) (Crispeels & Sadava, 1994).

Among the alkaloids, around 30 are used therapeutically and they have a broad spectrum of pharmacological effects. In spite of intensive research on in vitro production of these useful alkaloids for more than 20 years only, very few alkaloids like berberine have succeeded to the level of commercialization (Chrispeels & Sadava, 1994). The low producing ability of the cultured cells in most cases, is the main reason attributed for the low success in commercialization. Hence to increase their producing ability various approaches have been tried by many researchers in several systems.

Though ephedrine [1-phenyl-2-(methyl amino)-1-propanol] was discovered in 1887, it was Chen and Schmidt (1930) who brought out some of its hitherto unknown pharmacological properties, arising particularly from its relationship with adrenalin. Ephedrine and the related compounds pseudoephedrine and nor-ephedrine are adrenergic agents widely used in asthma, ophthalmic, cold and allergic products (Gal, 1984). Ephedrine is extensively used as a substitute for epinephrine against bronchial asthma of allergic and reflex types (Arya & Ramawat, 1988).

Ephedrine $C_{10}H_{15}ON$ is colourless, crystalline substance m.p. 41°C-42°C. The hydrochloride forms colourless needles m.p. 216°C (a) 15D = -34.2 in water and (a) 15D = -6.81 in absolute alcohol. The platinichloride crystallizes in colourless needles m.p. 186°C.

Chemical structure of ephedrine



1R, 2S – (–) Ephedrine

Pseudoephedrine or isoephedrine $C_{10}H_{15}ON$ occurs with ephedrine in *Ephedra gerardiana* and *E.intermedia* and is formed by heating ephedrine with hydrochloric acid (a) 15D = 50. It is a dextro rotatory isomer of ephedrine and melts at 114-115°C. The base is a white colourless crystalline substance occurring in the form of long needles freely soluble in alcohol. The hydrochloride forms colourless needles, m.p. 176°C.

The most important property of ephedrine is its stability, its solutions are not decomposed by light, air or heat and age apparently does not affect their activity. The great resistance of ephedrine to oxidation compared with epinephrine was demonstrated (Anon, 1972).

In pharmacological action, ephedrine is similar to adrenalin, but is more persistant. It is more stable to metabolic conditions and can be administered orally unlike adrenalin which has to be administered by injection. Ephedrine stimulates the

respiratory centre, increasing the depth of respiration, reinforces heart action and dialates the bronchi and hence its use in bronchial asthma. It also possess analeptic action, due to its central nervous stimulation, which is the basis for its use in the treatment of depression by drugs and for the release of narcolepsy. Ephedrine exerts a slight local anaesthetic action and this property seems to be greatly developed in the L-, & DL-forms of cinnamyl ephedrine. D-pseudoephedrine resembles ephedrine qualitatively, but its effect is on blood pressure and bronchi (Anon, 1972).

Ephedrine and its stereoisomer, pseudoephedrine are among the most commonly used naturally occurring drugs. The main natural source of ephedrine is *Ephedra* spp. The genus *Ephedra*, a group of xerophytic woody, perennial shrubs belongs to the gnetopsida, one of the most unusual classes in the plant kingdom. These plants are evolutionarily advanced gymnosperms with several angiosperm characteristics (Ovens & Hardev, 1990). Extracts of *Ephedra* have been used in traditional Chinese medicine for over five thousand years to treat asthma, nose and lung congestion, hay fever, malaria and several other ailments (Evans, 1989). The main active principles are the alkaloids, L-ephedrine (Eph) and d-pseudoephedrine (Peph). Ephedrine is close in activity and structure to adrenalin, elevating blood pressure, increasing heart rate and respiratory activity. Ephedrine and pseudoephedrine have an estimated retail market value worth US $ 100 million (Table 1.2). But the natural source of this alkaloid is not sufficient to meet the huge demand of this drug. Moreover, the indiscriminate collection of this species resulted in a drastic depletion of its natural source. At present, commercially, they are obtained by

Table 1.2
The estimated world market for selected plant products

Secondary metabolite	Estimated retail market value (million US $)
Medicinal	
Ajmalicine	5
Codeine	90-100
Corticosteroid	300
Ephedrine, Pseudoephedrine	100
Quinine	50
Vimblastine, Vincristine	50-75
Flavour & Fragrance	
Cardamom	25
Cinnamon	4-5
Spearmint	85-90
Vanilla	15
Agrochemicals	
Pyrethrines	20

* Chrispeels & Sadava (1994).

synthesis only. The synthetic compounds are comparatively very cheap since the highly active purified natural compound is expensive. The pure ephedrine costs US $ 869 per kg and pseudoephedrine costs US $ 1317 per kg (Sigma Catalogue, 1997). Although limited success was achieved with cultured tissues, there is a need to develop high yielding clones of *Ephedra* spp. for cultivation and a suitable bioprocess for *in vitro* production of ephedrine (Arya & Ramawat, 1988).

In vitro callus culture of both low and higher alkaloid yielding species of *Ephedra* was attempted by various workers (Ramawat & Arya, 1979a; Bhatnagar & Singh, 1984). Although callus culture of different species of *Ephedra* was successful, only trace quantities of ephedrine and pseudoephedrine could be produced by *in vitro* culture and, the ability to produce alkaloid diminished to zero with successive subcultures (O'Dowd *et al.*, 1993). In general, reports on the successful suspension culture of gymnosperms are only very few (Teasdale *et al.* 1986).

The plant cell culture technique offers an attractive alternative for the production of secondary metabolites, but one of the main problems with plant cell cultures, as a source of phytochemicals, is that the cultures, initially show a productivity which is far below that needed to make any commercial exploitation economically feasible (Yeomann *et al.* 1990). But a number of strategies like induced mutagenesis and selection of high yielding cell lines, inducing stress factors, addition of specific elicitors and precursors, use of permeation agents etc. have been identified which can positively influence the productivity in cell culture (Rokem & Goldberg,

1985; Johnson, 1993; Rajendran, 1994).

The preferred approach for secondary metabolite production in cell cultures for maximum yield is the use of organised or differentiated cultures. The organisation is frequently associated with improved production of desired compounds. The concept of organisation and differentiation has led to the use of immobilization technology in plant cell culture system which has long been used for microbes and enzymes. Immobilization of plant cells ie. physically restraining the cells in/or on fixed support can be seen as an intermediate stage between homogenous suspension cultures and the highly organised tissue matrix of whole plant (Yeoman *et al.* 1990). This led to enhanced production of secondary metabolites in immobilized cell systems.

Many methods of immobilizing plant cells have been developed, viz., entrapment in natural polymers like gels or in nets or foam, immobilization in hollow fibre membranes etc. Among them, entrapment with calcium alginate is one of the simplest methods of immobilizing cells and it has found widespread use in laboratory and pilot scale studies (Brodelius, 1990).

Immobilized plant cells sometimes induce or increase secondary metabolite formation and may even stimulate excretion of secondary metabolites. The explanation for this phenomenon is that enclosure in a support exerts a certain stress on the plant cells leading to restricted growth, the conditions generally considered as essential for secondary metabolite production (D'Souza, 1989). This restricted growth may simply be due to lack of space in the support or as a result of relatively slow mass transfer

processes caused by depletion of substrates and/or accumulation of growth inhibitor complexes (D'Souza, 1989).

In the light of the very huge demand for natural ephedrine and pseudoephidrine, a search for an angiosperm plant containing the alkaloid ephedrine was made and could locate *Sida* spp. of malvaceae family. Sida is a large genus of, herbs and shrubs distributed throughout the tropics. About a dozen species occur in India. The medicinally important species known are S.*rhombifolia* *S.cordata* and *S.spinosa* (Anon, 1972). Among the various species, *S.rhombifolia* is the most widely used one in the traditional system of medicine. An attempt was made in the present study to develop an ideal bioprocess for the in vitro production of ephedrine from the cell culture system of *Sida rhombifolia* Linn. ssp. *retusa*. The callus and suspension culture were initiated and attempts were made to enhance the yield positively by employing various strategies like mutagenesis, immobilization and addition of precursors, elicitors and permeabilizing agents.

CHAPTER II

Review of literature

2.1 Plant cell culture as source of secondary metabolites

The plant cell culture offers a potential alternative for phytochemicals which are of importance to food and pharmaceutical industries (Barz & Ellis, 1987; Fowler, 1987; Furuya, 1988; Fowler & Stafford, 1992). But the plant cell cultures are subject to the vagaries of somaclonal variations which may result in loss of productivity with culture age. To overcome this problem, several alternatives were explored which resulted in the use of organised or semiorganised tissue and high yielding stable hairy root cultures. The availability of hairy root cultures obtained by genetic transformation with *Agrobacterium rhizogenes* (Tepfer, 1984) has renewed interest in this system. Hairy root cultures can grow as fast as unorganised plant cell suspension while maintaining the stability of yields. Furthermore, hairy roots can express root specific metabolic pathways as efficiently as normal roots (Flores & Filner, 1985; Hamill *et al.* 1987; Flores & Curtis, 1992). These cultures have shown long-term stable production of many secondary metabolites (Flores *et al.* 1987; Signs & Flores, 1990). Hairy root cultures are also capable of transforming inert xenobiotics into bioactive metabolites (Flores & Curtis, 1992). Some hairy roots when exposed to light turn green and can be grown photoautotrophically (Flores *et al.*, 1993), and these photoautotrophic roots show much higher levels of total alkaloids than the dark grown roots.

Use of immobilized plant cells for higher productivity and continuous production of metabolites and biotransformation of exogenous substrates into desired compounds have also been a topic of interest. There have been indications of potentials in diagnostics and related areas, through the use of cell culture derived plant enzymes (Stafford & Fowler, 1991). Production of macromolecule – ribosome inactivating proteins (RIP) such as trichosanthin for AIDS treatment (Flores, 1992) and production of taxol from *Taxus brevifolia* for cancer therapy have been possible by cell cultures (Fett Neto *et al.*, 1992 & 1993).

2.2. Strategies to improve secondary metabolite production

Even though plant cell culture technique offers an attractive alternative for the production of secondary metabolites, one of the key problems with plant cell cultures as a source of phytochemicals is that the cultures initially show a productivity which is well below that needed to make any exploitation economically feasible (Berlin, 1986; Yeoman *et al.*, 1990), except few cases like accumulation of rosmarinic acid in *Coleus blumei* and glutathione in *Nicotiana tabacum* cultures, where the cultures produce nine times and ten times respectively more than the plant (Fowler, 1986). Thus, in recent years, much effort has been put into the development of methods to enhance the productivity by plant cells. A number of strategies have been identified by various researchers which can potentially influence the productivity in plant cell culture systems apart from optimization of media and cultural conditions. These include

a) induced mutagenesis and selection of high yielding cell lines

b) inducing stress by nutrient limitation

c) presence of specific elicitor compounds

d) supply of precursors

e) use of permeation agents

f) immobilization technique

2.2.1 Induced mutagenesis and selection of high yielding cell lines

The genetic instability of higher plant cells cultivated *in vitro* (Mitra & Steward, 1961) and its transfer to regenerate was reported (Heinz & Mee 1969). The field of cell culture mutants has been reviewed by a number of authors (Widholm, 1983; Wersuhn, 1989).

The advantages of using toxic agents for selection is that majority of the wild type cells are killed, while the desired variant survives the treatment. A high serpentine yielding cell line of *Catharanthus roseus* was obtained after X-ray irradiation (Dues, 1978). Further, a fine high yielding stable strain of *Anisodus acutangulus* with 30% higher yield of scopolamine was derived through irradiation with 4000 R of X-rays (Guang-zhi *et al.* 1982). A cell line of *Lavendula vera* producing a high level of free biotin was obtained by gamma ray irradiation at 10 KR dose (Wataneba *et al.* 1982). The selection of UV tolerant

cell lines of *Nicotiana* and *Anchusa* which exhibited higher level of phenolics was reported (Quesnel & Ellis, 1989).

The interest in amino acid analogues began with the isolation of the p-fluorophenyl alanine (PFP) tolerant tobacco cell lines with ten fold increased levels of cinnamoyl putriscenes (Palmer & Widholm, 1975). However, PFP tolerant *Daucus* and *Catharanthus* cells did not show any overproduction of phenolic compounds (Palmer & Widholm, 1975).

Amino acid analogues have been often used for establishing cell lines with higher yield of secondary metabolites (Berlin, 1990). Induction of a mutant having altered permeability could also be important, because plant cells generally accumulate their metabolites intracellularly. This is very disadvantageous in commercial production because the amount of compounds produced is limited to a low level. Unfortunately, the secretion mechanisms for secondary products in higher plant cells have not yet been elucidated and extensive fundamental studies are required before meaningful manipulation can take place. Furthermore, the biosynthetic pathways of many secondary metabolites and their regulation mechanisms in higher plants are obscure, therefore, it is also difficult to decide what kind of mutants should be induced in order to increase production ability.

2.2.2 Inducing stress by nutrient limitation

The plant cell cultures are usually grown heterotrophically, with a simple sugar as carbon source along with inorganic source of other nutrients. In general, sucrose appears better than glucose or fructose for secondary product formation (Dougall, 1980). An inverse relationship between growth and product yield exists and products tend to accumulate at the end of growth phase (Johnson, 1993). Therefore, the general approach has been to slow down the growth by limiting the supply of sugar, nitrogen or phosphorus (Johnson, 1993). Further, the cultural conditions which promote high rate of cell division are commonly not conducive to a maximum rate of secondary product formation (Rokem & Goldberg, 1985). Low phosphate levels stimulated cinnamoyl putrescine accumulation in *Nicotiana tabaccum* cell suspension by 3-4 fold (Knobloch & Berlin, 1981; Schiel *et al.*, 1984). Anthraquinone formation in *Morinda citrifolia* is sensitive to level of nitrate in the medium being optimal at 25-40 mM (Zenk *et al.*, 1975). More than ten fold increase of capsaicin from immobilized cell cultures of *Capsicum* sp. was noted when nitrate was eliminated from the culture medium (Johnson, 1993).

2.2.3 Presence of specific elicitor compounds

A relatively new approach for the production of secondary metabolites from plant cells is the use of biotic and abiotic elicitors. The induction and accumulation of antibiotics and chemicals in normal intact plants after microbial insult is a well established phenomenon (Dicosmo & Tallevi, 1985; Dicosmo & Misawa, 1985) and certain molecules or elicitors usually associated with the surface of microorganisms are known to stimulate

secondary metabolic pathways in plant cells (Eilert, 1987). The best characterized biotic elicitor substances are the fungal elicitors, various of which have been identified from fungal wall materials as glucan polymers and glycoproteins and abiotic elicitors like UV-irradiants, salts of heavy metals and chemicals including polylysine (Dixon, 1980; Albersheim & Darvill, 1984).

Elicitation of phytoalexins, has opened a new avenue for the production of secondary metabolites (Dicosmo & Misawa, 1985). Elicitors used for enhancement of secondary metabolites have been well investigated. Table 2.1 shows the list of potential biotic elicitors and the elicited metabolites. The stimulation of alkaloids, codeine and morphine by fungal spores in *Papaver somniferum* cultures has been reported (Heinstein, 1985). Autoclaved fungal mycelia induced the accumulation of diosgenin in *Dioscorea deltoidea* cultures (Rokem *et al.*, 1984). The production of berberine and shikonin was enhanced in cultured cells treated with fungal extracts (Funk *et al.*, 1987; Kim & Chang, 1990). The elicitor preparation from *Fusarium conglutinans* enhanced thiophene production in *Tagetes* spp. (Mukundan & Hjortso, 1990) and solavetivone production was increased by *Rhizoctonia solanii* in *Hyoscyamus muticus* (Ramakrishna *et al.* 1993). Increased levels of *ajmalicine* and *catharanthine* in *Catharanthus roseus* (Dicosmo *et al.* 1987; Asada & Shuler, 1989) capsaicin production in *Capsicum annum* (Johnson, 1993). and anthocyanin production in *Daucus carota* (Rajendran *et al.* 1994) by fungal elicitors have been documented.

Tables 2.1

List of potential biotic elicitors*

Secondary metabolite	Plant species	Elicitor (Mycelial extracts or culture filtrates)
Thiophene	*Tagetus patula*	*Fusarium conglutinans F.oxysporum*
Sanguinarine	*Eschscholtzia*	*Penicillium sp.*
Diosgenin	*Dioscorea deltoidea*	*Rhizopus arrhizus*
Benzofurans	*Ageratiana adenophora*	*Saccharomyces cerevisiae*
Berberine	*Thalictrum rugosum*	*S.cerevisiae*
Tropane alkaloid	*Datura stramoneum*	*Phytophthora megasperma*
Ajmalicine	*Catharanthus roseus*	*Micromucor isobellina*
Furanocoumarins	*Petroselinum hortense*	*Alternaria carthami*
Medicarpin	*Cicer arietinum*	*Ascochyta rabiei*
Phytuberin	*Nicotiana tabacum*	*Pseudomonas solanacearum*
Capsaicin	*Capsicum annum*	*Gliocladium deliquescens*
Anthraquinone	*Morinda citrifolia*	*Aspergillus niger*

*Rajendran (1994).

Apart from fungal elicitors, preparations from yeast and bacteria were also reported to enhance secondary metabolites. In addition to biotic elicitors, compounds of non-biological origin act as elicitors. They include metal ions, chemically defined compounds and physical agents such as UV light (Lee *et al.* 1981; Darvill & Albersheim, 1984). Of these, metal ions were reported widely to induce secondary metabolite production (Threlfall & Whitehead, 1988a; 1988b). The elicitor activity of calcium and several calcium dependent processes have been reported in many systems (Kurosaki *et al.* 1987; Stab & Ebel, 1987;).

The mechanism by which elicitors enhance secondary metabolism remains to be clearly defined and much current research activities are directed towards elicitor-mediated biochemical processes. Indirect studies on the elicitor treated plasma membrane of cells reveal the presence of elicitor specific receptors (Stossel, 1984). Toppan & Esquerre-Tugaye (1984) postulated that fungal glycoproteins and carbohydrate elicitors may bind to receptor sites on plasma membrane and elicit a phytochemical response. Elicitors are believed to act by ionic communication which can be brought about by changes in pH, electrolyte leakage, depolarization or inhibition of electrogenic ion pump (Kota & Stelzig, 1977; Katou *et al.* 1982). For example, the abiotic elicitor vanadate is known to inhibit cationic pumping of ATP ases (O'Neill & Spanswick, 1984), which indirectly influence the intracellular Ca level (Macara & Gray, 1987).

The possibility of the intermediary second messengers or response couplers to transit the elicitation signal intracellularly in order to effect changes in host gene expression or other metabolic manifestation of elicitation has been studied (Dicosmo & Misawa, 1985). In this context, studies on the role of calcium-calmodulin, polyamines and cyclic AMP have been reported (Dixon, 1986). The involvements of calcium and its protein calmodulin were found in several systems from the studies using inhibitors or modulators of this process (Kurosaki *et al.* 1987; Ragothama *et al.* 1987). The use of fungal and abiotic elicitors, in the induction of enzyme activity has been shown to increase product yields in many systems. During studies on PAL, however, no direct relationship between enzyme activity and product formation was found to exist (Jones, 1984).

2.2.4 Supply of precursors

Under some conditions, the flux through a particular biosynthetic pathway may be limited by the availability of precursors or particular metabolic intermediates (Johnson, 1993) and supply of these precursors, externally, increased the rate of product biosynthesis. Generally, the greater the number of steps between the precursor and the product, lower the yield. However, when single step biotransformations are considered, conversion was very efficient, often approaching 100% (Johnson, 1993).

The earliest study of biotransformation using immobilized plant cells was the 12-B-hydroxylation of digitoxin to digoxin by *Digitalis lanata* entrapped in alginate (*Brodelius et al.*, 1979). Alfermann *et al.* (1983) reported β-Methyl digitoxin hydroxylation to β-

methyl digoxin. *Daucus carota* cells immobilized in alginate are capable of hydroxylating digitoxigenin to periplogenin, eventhough neither of these compounds are found in *D.carota* (Jones & Veliky, 1981). *Mucuna pruriens* cells entrapped in alginate were used for biotransformation of L-tyrosine to L-DOPA (Wichers *et al.*, 1983). Brodelius and Nilsson (1980 and 1983) extensively studied the synthesis of ajmalicine isomers from relatively distant precursors, tryptamine and secologanin by immobilized *Catharanthus roseus* cells.

Yeoman *et al.* (1980) showed that the production of *capsaicin* by cells of *Capsicum frutescens* could be increased significantly by supplying 5 mM isocarpic acid, which is only a few steps away from the product. On the other hand, the addition of phenyl alanine, many steps distant from *capsaicin*, also increased the product yield but to a smaller extent (Lindsey & Yeoman, 1984).

2.2.5 Use of permeation agents

In most cases, the products formed by plant cells are stored in the vacuoles. Only a few compounds are released spontaneously by plant cells grown in culture which are listed in Table 2.2. A wide variety of approaches such as treatments with organic solvents, electropermeabilization, ultrasonication etc. are known, which permeabilize cells and cause release of products (Felix, 1982).

Table 2.2

List of secondary products excreted into the extracellular compartment*

Plant species	Secondary metabolite
Datura innoxia	Atropine
Coptis japonica	Berberine
Thalictrum minus	Berberine
Coffea arabica	Caffeine
Coffea robusta	Caffeine
Cinchona ledgeriana	Quinine
Lupinus polyphyllus	Lupanine
Nicotiana tabacum	Nicotine
Macleaya microcarpa	Protopine
Papaver somniferum	Sanguinarin
Datura innoxia	Scopolamine
Datura innoxia	Naphthoquinone
Lithospermum erythrorhizon	Shikonin
Capsicum fruitescens	Capsaicin
Thuja occidentalis	Monoterpenes

*Barz *et al.* (1990).

In order to release products from vacuoles of plant cells, two membrane barriers viz. plasma membrane and tonoplast, have to be penetrated. The permeability can be monitored by measuring the activity of five enzymes of primary metabolism viz., hexokinase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, malic enzyme and citrate synthase (Brodelius, 1988). Apart from organic solvents, some polysaccharides such as chitosan can also be used as a permeabilizing agent (Young & Kauss, 1983; Knorr & Teutonico, 1986). It was also reported that under the influence of chitosan release of oxalate from immobilized cells of *Amaranthus tricolor* was enhanced. Chitosan served as both immobilizing and permeabilizing agent. Other permeabilization methods include ultrasonication, electroporation and ionophoretic release in which the cells are subjected to a low constant current in a specially designed device (Brodelius *et al.* 1988). Laminarin, a plant polysaccharide, was reported as effective cell permeabilizing agent (Johnson, 1993).

However, release of products by using polysaccharides such as chitosan and laminarin are preferred rather than toxic chemicals like DMSO when food, cosmetic and pharmaceutical products are concerned (Johnson, 1993).

2.2.6 Immobilization technique

The use of immobilized plant cells offers distinct advantages for the production of secondary metabolites by plant cell cultures (Shuler, 1981; Brodelius, 1985). The immobilization of plant cells has been developed as an alternative to suspension cultures to

provide cell to cell contact and favourable cell differentiation to improve secondary metabolite production (Johnson, 1993).

The primary limitation of plant cell culture is that the product must leach out to the exterior or be extracellular. Most of the plant cell products are located in vacuoles and their excretion can be effected by using permeability agents and altering the pH. This technique has been successfully used to enhance secondary metabolites such as capsaicin (Lindsey & Yeoman, 1985; Johnson *et al.* 1990), anthraquinone (Brodelius *et al.*, 1980) and berberine (Kobayashi *et al.*, 1987). Further, this technique is highly advantageous in effecting biotransformations in plant cell cultures.

2.3 Medicinal compound production by plant cell cultures

Studies on the intensive screening of plants for medicinal compounds, particularly antitumor agents originated during 1950's with the discovery of two alkaloids vinblastine and vincristine from *Catharanthus roseus* followed *by Podophyllotoxin* from *Podophyllum peltatum* (Hartwell, 1976). Although much efforts have been made to produce them by cell culture, it could not be commercialized so far (Roja & Heble, 1994).

Production of various types of alkaloids by plant cell suspension cultures have been reported (Teshima *et al.* 1988; Nair *et al.* 1992; Enaksha *et al.* 1993; Ketchum *et al.* 1995; Ketchum & Gibson, 1996). Some of the alkaloids have potent antineoplastic activity and intensive research for *in vitro* production of such compounds are in progress

because of their high value and great demand. The anticancer compounds isolated from plants include taxol, campothecin, holacanthone, bruceanthin ellipticine, maytansine, homoharringtoxine, indicine-N-Oxide, triptolides etc. (Suffness & Douros, 1982). Campothecin and taxol are secondary metabolites found respectively in the wood bark of *Campotheca acuminata*, a native to China and *Taxus brevifolia* found in the North West Pacific Coastal Region of the United States (Wall & Wani, 1995). Campothecin and 9-methoxy derivatives were isolated from the roots, bark and leaves of tree *Nothapodytes foetida*, an indigenous tree abundant in Western Ghats of India (Govindachari & Viswanathan, 1972). Culture of *N.foetida* was initiated and higher levels of 9-methoxy campothecin was detected in the differentiated cultures in comparison with the callus cultures (Roja & Heble, 1994).

Castanospermine, a tetrahydroxy indolizidine alkaloid from *Castanospermum australe* was found to inhibit the replication of HIV (Molyneux, 1990). The cultures of *C.auestrale* synthesized high levels of castanospermine than the intact mature tree (Roja & Heble, 1995).

2.4 Natural sources of ephedrine

Several species of *Ephedra* contain the alkaloidal amine "ephedrine". The genus *Ephedra*, a group of xerophytic woody, perennial shrubs belongs to gnetopsida, one of the most unusual classes in the plant kingdom (O'Dowd *et al.* 1993). These plants are

evolutionarily advanced gymnosperms with several angiosperm characteristics (Owens & Hardev, 1990). The genus is scattered all over the world and is found in the Mediterranean, the Himalayas, the Andes and the Rocky Mountains from Chile to California (Arya & Ramawat, 1988).

Three species of *Ephedra* (*E.sinica, E.equisetina, E.intermediae*) occur in Northern and North Western China. The important species of *Ephedra* which occur in India include *E.gerardiana* Wall. (Syn. *E.vulgaris* Hook; *E.major* Host; *E.nebrodensis* Tineo) and *E.intermedia* Schrenk & Mey (Syn. *E.pachyclada* Boiss). The alkaloid content of different species of *Ephedra* and other species containing ephedrine are presented in Table 2.3.

It is clear that Indian *Ephedra, E.gerardiana* Wall (Synonyms, *E.nebrodensis* Tineo, *E.major* Host, *E.vulgaris* Rich), *E.intermedia* Schrenk & Meyer (Syn. *E.pachyclada* Boiss) (Satyavati *et al.* 1976) and Chinese *Ephedra, E.sinica* Stapf. And *E.equisetina* Bunge (Hu, 1969) are rich sources of ephedrine.

China has been the principal source of supply of *Ephedra*. Though some of the Indian species contain comparatively high percentage of ephedrine, there was demand for Indian *Ephedra* in the International markets only when supplies from China and Spain were difficult to obtain (Anon, 1972). The principal source of supply of *Ephedra* in India, before partition, was Baluchistan. *Ephedra* from Lahul, Pangi and Kashmir is suitable for

Table 2.3

Alkaloid content of various *Ephedra* spp. and other species containing ephedrine

Species	Origin	% ephedrine (dwb*)	Reference
Ephedra			
E.gerardiana	India	1.70	Ramawat & Arya 1979a
E.gerardiana	India	2.15-2.79	Chopra *et al.* 1956
E.nebrodensis	Pakistan	1.30	Shah & Shah, 1966
E.foliata	India	0-trace	Chopra *et al.* 1956
E.foliata	India	Trace-0.01	Ramawat & Arya 1979a
E.sinica	China	1-2.5	Hu, 1969
E.distachya	Europe	Ephedrine	Cromwell, 1955
Catha edulis		d-norephedrine	Cromwell, 1955
Taxus bacata		Ephedrine	Cromwell, 1955
S.acuta		Ephedrine	Anon, 1972
S.cordifolia		Ephedrine	Anon, 1972
S.rhombifolia		Ephedrine	Anon, 1972

* dwb – dry weight basis

commercial exploitation. Foreign markets demand *Ephedra* containing more than 1% ephedrine (Anon, 1972). Since partition, however, supplies from Baluchistan have become uncertain and ephedrine production in India has fallen. Further, the indescriminate collection of *Ephedra* spp. might have resulted in a drastic depletion of its natural source.

The *Sida* spp. belonging to m*alvaccae* family was also reported to contain ephedrine and pseudoephedrine (Anon, 1972; Nair *et al.* 1992; Sankar & Nair 1997). The total ephedrine content on whole plant basis of *S.cordifolia Linn.* was reported to be 0.09%, the seeds contain the maximum amount (0.28%) (Anon, 1972). Four alkaloids were reported to occur in the aerial parts of *S.acuta Linn.* Seeds contain 0.26% of alkaloids and roots 0.07%. The major alkaloid present is ephedrine (Anon, 1972). The leaves of *S.rhombifolia Linn.* was reported to contain ephedrine. Roots contain 0.05% of alkaloids, one of which is ephedrine (Anon, 1972).

2.5 Cell culture studies on ephedrine production

Cell culture studies on *Ephedra* spp. were tried by various researchers. *E.foliata*, a widely scattered and available species in India, was cultured *in vitro* for demonstration of the regenerative potential of female gametophyte (Sankhla *et al.* 1967), culture of male and female gametophytes (Konar & Singh, 1979; Singh *et al.* 1981) and determination of the amino acid content (Uddin, 1977). Ramawat & Arya have studied the morphogenesis in callus (1976), the carbohydrate nutrition and metabolism (1977), the *in vitro* ephedrine

production in *E.foliata* and *E.gerardiana* (1979a,b&c) and the nitrogen nutrition and its effect on protein content (1980). Strans and Gerding (1963) used *Ephedra* tissues to study the indole acetic acid and oxidase enzyme activity.

The maximum amount of ephedrine (0.3%) was obtained in callus tissues grown in medium containing kinetin and indole butyric acid (Ramawat & Arya 1979b). Light stimulated the production of alkaloid in *E.gerardiana* callus (Ramawat & Arya 1979a). The maximum yield of ephedrine was recorded in callus tissues grown on MS medium supplemented with 0.1 g l^{-1} *L.phenylalanine* (Ramawat & Arya 1979c). A synergistic effect of 1BA and L-phenylalanine and *DL-methionine* was observed on ephedrine yield of callus cultures (Ramawat & Arya, 1979c).

Callus culture of low alkaloid yielding species (trace – 0.01% dwt. alkaloid) *E.foliata* (Konar & Singh 1979; Ramawat & Arya, 1980; Shukla, 1980; Bhatnagar & Singh, 1984) and higher alkaloid yielding species (0.8-2.5% alkaloid; 1:1 Eph:Peph) *E.gerardiana* were reported (Ramawat & Arya 1979a, 1979b, 1980). Though the batch suspension culture of *E.foliata* was reported, no details were offered of the culture growth characteristics (Uddin, 1977).

However, O'Dowd *et al.* (1993) examined in detail, the callus production, suspension culture and *in vitro* alkaloid production of *Ephedra* spp. Among the various *Ephedra* spp. examined, neither the parent plants nor the *in vitro* cultures of *E.distachya*,

E.fragilis and *E.sexatilis* produced alkaloids. Trace quantities of L-ephedrine and trace (0-0.14% dwt.) L-pseudoephedrine were produced by *in vitro* cultures of *E.andina, E.equisetana E.gerardiana, E.intermedia, E.major* ssp. *procera* and *E.minima*. But the ability to produce ephedrine diminished to zero in successive subcultures. They attributed the reason that the ephedrine production in *Ephedra* spp. is associated with the organisation of the tissues. Further, the gymnosperm nature of *Ephedra* spp. was also considered as responsible for the low success in suspension culture.

In *Sida* spp. only two reports are available regarding the *in vitro* culture for ephedrine production. Induction of calli in *Sida* spp. to screen the calli for *in vitro* production of ephedrine (Sankar & Nair, 1997) and callus induction and *in vitro* growth profile of *S.cordifolia* callus cultures (Nair *et al.* 1992). Whereas no information is available in the literature regarding the bioprocess optimization for ephedrine synthesis under cell suspension culture of *Sida* spp.

2.6. Scope of the present study

The great demand (worth US $ 100 million) for natural ephedrine, the inadequate availability of its natural source for extraction and comparatively poor performance of the *Ephedra* ssp. in *in vitro* cell culture system have suggested the need for *in vitro* production of ephedrine from an alternate source. Hence, in the present study a search for an alternate source was made and selected *Sida* spp. of *malvaceae* family as a potential

and alkaloid production profile of *S.rhombifolia Linn. ssp. retusa* callus and cell suspension culture with a view to develop a suitable bioprocess for *in vitro* ephedrine production.

2.7 Objectives of the present study

The specific objectives of the study include the following:

1. To initiate callus culture of *S.rhombifolia* Linn ssp. *retusa* and to optimize the media and cultural conditions for better callus growth and ephedrine production.

2. To initiate and establish suspension culture.

3. To enhance the alkaloid yield by (a) permeabilization (b) stress induction, (c) elicitor induction, (d) precursor feeding and (e) mutation.

4. To immobilize the cells and evaluate the production pattern.

5. To isolate and characterize the alkaloid.

CHAPTER III

Studies on callus initiation *in Sida rhombifolia* Linn. ssp. *retusa* (Linn.) Borssum

3.1 Introduction

Ephedrine [1-phenyl-2-(methyl amino)-1-propanol], the main alkaloid of many *Ephedra* spp. is an orally active sympathomimetic agent. Compared to adrenalin, it is weaker but longer acting. It has wide application in clinical use against bronchial asthma, hay fever, wooping cough, myasthenia gravis, dysmenorrhea and heart block (Stockes-Adam Syndrome).

Presence of this alkaloid is also reported in *Aconitum napellus, Catha edulis, Taxus bacata, Sida cordifolia, Roemaria refracta* and some other species of Ephedraceae, Ranuncluaceae, Celastraceae, Taxaceae, Malvaeeae and Papaveraceae. Although *in vitro* alkaloid production of various species of *Ephedra* has been tried (O'Dowd *et al.*, 1993), only few species of Ephedra like *E.gerardiana, E.intermedia, E.equisitana & E.major* produced trace quantities of ephedrine and pseudoephedrine by *in vitro* cell culture system. But they have reported that the cell culture system of *Ephedra* species is not good for *in vitro* production of ephedrine, as the ability to produce the alkaloid diminished drastically in subsequent subcultures. The unsatisfactory results obtained in the *in vitro* culture of *Ephedra* spp. may be due to its gymnosperm nature, as suggested by Teasdale *et al.* 1986.

Considering the huge demand for natural ephedrine, a search for an angiosperm plant containing ephedrine, as an alternate to *Ephedra* spp. was made and the *Sida* species of malvaceae family could be identified (Anon 1972; Nair *et al.* 1992). In the present study, the most widely used *Sida* species in the traditional system of medicine viz. *Sida rhombifolia* Linn. ssp. *retusa* (Linn.) Borssum and another related species viz. *Sida acuta* Linn. were evaluated for ephedrine production under *in vitro* conditions.

3.2 Material and methods

3.2.1 Taxonomic position

Sida, a large genus of herbs and shrubs, belongs to the family malvaccae and distributed throughout the tropics. Some of the plants are medicinally important and yield fibre. Although about a dozen species occur in India, the medicinally important species known are *S.rhombifolia*, *S.acuta*, *S.cordifolia*, *S.cordata* and *S.spinosa* (Anon, 1972).

Sida acuta

An errect, perennial undershrub or shrub 1.5m high, is distributed throughout the hotter parts of India. Bark smooth, greenish, root thin, long, cylindrical, very rough, contorted; leaves lanceolate, linear-lanceolate, obovate-lanceolate or lanceolate-oblong, glabrous, flowers yellow, solitary or in pairs; seeds smooth and black.

This species is not only an important source of medicine but also yields good fibre. The leaves possess demulcent and diuretic properties and are used for treatment of rheumatism. Root is bitter and said to possess astringent, tonic, stomachic, diaphoretic and antipyretic properties. Besides, it is useful in nervous and urinary diseases, disorders of blood and bile and in chronic bowel complaints.

Four alkaloids are reported to occur in the aerial parts of the plant and three in the roots. Seeds contain 0.26% of alkaloids and roots 0.07%. The major alkaloid present is ephedrine (Anon, 1972).

Sida rhombifolia

An erect, very variable, minutely hairy, branched undershrub with a firm woody stem and intricate branches; leaves short petioled, obovate, truncate or more often retuse and serrate; flowers yellow, solitary and axillary; fruits enclosed within the persistent calyx, separating into one seeded cocci; seeds black and smooth.

This species is polymorphic, comprising a number of varieties. All the varieties are said to be medicinally important. They are good for rheumatism, flatulence, colic haemothermia, arthritis and diarrhoea. Leaves are reported to contain ephedrine. Roots contain 0.05% of alkaloids one of which is ephedrine.

3.2.2 Source of plant material

The *S.rhombifolia* Linn ssp. *retusa* (Linn.) Borssum and *S.acuta* Linn. plants were collected from the Aromatic and Medicinal Plants Division of the Kerala Agricultural University, India and were grown in pots kept in a green house. On maturity, the whole plants were uprooted and different plant parts viz. roots, stem, leaves and fruits were separated. After recording the fresh weight they were dried in an oven at 60°C until two consecutive weights were equal. The dried material was powdered in a mixer grinder (Sumeet, India) and used for extraction.

3.2.3 Initiation of callus cultures

3.2.3.1 Media preparation

Murashige and Skoog (1962) medium with 3% sucrose, 0.8% agar and 100 mg l^{-1} myoinositol was used as the basal medium. 2,4-Dichlorophenoxy accetic acid (2,4-D), α-naphthalene accetic acid (NAA), indole 3-accetic acid (IAA), 6-benzyl aminopurine (BAP) and 6 furfuryl aminopurine (Kinetin) were tried as plant growth regulators. All the chemicals, reagents and media used were of tissue culture grade and purchased either from Sigma Chemicals or Hi Media, India. For media preparation, all the components were added and pH adjusted to 5.6-5.8 prior to autoclaving.

3.2.3.2 Choice of explants

Shoot tips (3 mm), leaf segments (8mm x 5 mm pieces), nodal segments, internodal segments and petioles (5mm each) were tried as explants.

3.2.3.3 Surface sterilization, inoculation and incubation

After cleaning with soapy water, the explants were washed thoroughly with sterile distilled water, immersed in micanazole (20 mg l^{-1}) solution for 10 minutes. The segments were then rinsed 3-5 times with sterile distilled water followed by complete immersion in 70% ethanol for 15 seconds and then in 0.1% mercuric chloride (1% Tween-20 as surfactant) for 5-10 minutes. These sterilized segments were then rinsed 3-5 times with sterile distilled water.

The sterilized explants were then transfered to the prepared sterile solid culture medium aseptically and incubated at 25±2°C, and under illumination of 1000 lux (16h photoperiod).

3.2.3.4 Callus subculturing

The initiated calli were allowed to grow for about 4 weeks, then isolated and was maintained on the same medium by regular subculturing at 3 weeks interval.

3.2.4 Extraction procedure

One gram of the dried plant material/fresh weight of callus tissue was ground well with neutralised sand (100 mg) and moistened with 0.1N HCl in a mortar and pestle and homogenized in a homogenizer (Remi Motors, India). The homogenized tissue was then extracted with 10 ml methanol at alkaline pH (pH was adjusted to 10 with ammonia solution). The extraction was repeated thrice and the extract was centrifuged at 5,000 rpm for 10 minutes. The supernatant was pooled, vacuum dried

and the residue redissolved in known aliquots of methanol and used for ephedrine analysis.

3.2.5 Detection of ephedrine

Normal phase thin layer chromatography (TLC) was employed using 0.2 mm thick silica gel G60 F254 aluminium backed plates (Merck), and a solvent system including butanol, glacial acetic acid and water (4:1:1) and localisation agent - ninhydrin (O'Dowd *et al.*, 1993). The identification of the compound was made by comparing the R_f values with the standard ephedrine (Aldrich Chemicals, USA).

3.2.6 Assay of ephedrine

Ephedrine was assayed by spectrophotometry (Lykova 1987; Sethi 1993; Zhan *et al.* 1995 & Sayin *et al.* 1996). One ml saturated solution of sodium hydrogen carbonate was added to 3 ml extract and after the carbon dioxide evolution was stopped, added 1 ml of 2% (w/v) sodium metaperiodate, vortexed and kept for 15 min. at room temperature (28±2°C). It was then extracted with 5 ml of n-hexane (thrice) and the n-hexane extract was pooled and measured the absorbance at 242 nm in a spectronic 5 genesys spectrophotometer using n-hexane as blank. The method was based on the alkaloid oxidation by sodium metaperiodate and the formation of benzaldehyde (Sethi, 1993).

3.2.7 Statistical analysis

The experimental results were analysed by completely randomized design analysis (Compton, 1994).

3.3 Results

3.3.1 Choice of plant species

The presence of ephedrine in the plant/callus exctract was detected by the presence of a purple spot corresponds to the R_f value 0.56 in comparison with pure ephedrine (Plate 1). Initially the ephedrine content in different plant parts was evaluated in both the selected species viz. *S.rhombifolia* Linn. ssp. *retusa* and *S.acuta* Linn. Among the two species, *S.rhombifolia* contained more alkaloid compared to *S.acuta* (Fig.3.1). However, on analysis of individual parts, it was observed that fruits contained maximum alkaloid (2.9 mg/gdw and 2.00 mg/gdw respectively) for *S.rhombifolia* and *S.acuta* followed by leaves (2.5 mg/gdw and 1.8 mg/gdw respectively) in both the species. There was no significant difference between alkaloid contents of fruits and leaves in both the species. On comparison, *S.rhombifolia* was observed as a better source for *in vitro* production of ephedrine (Plate 2).

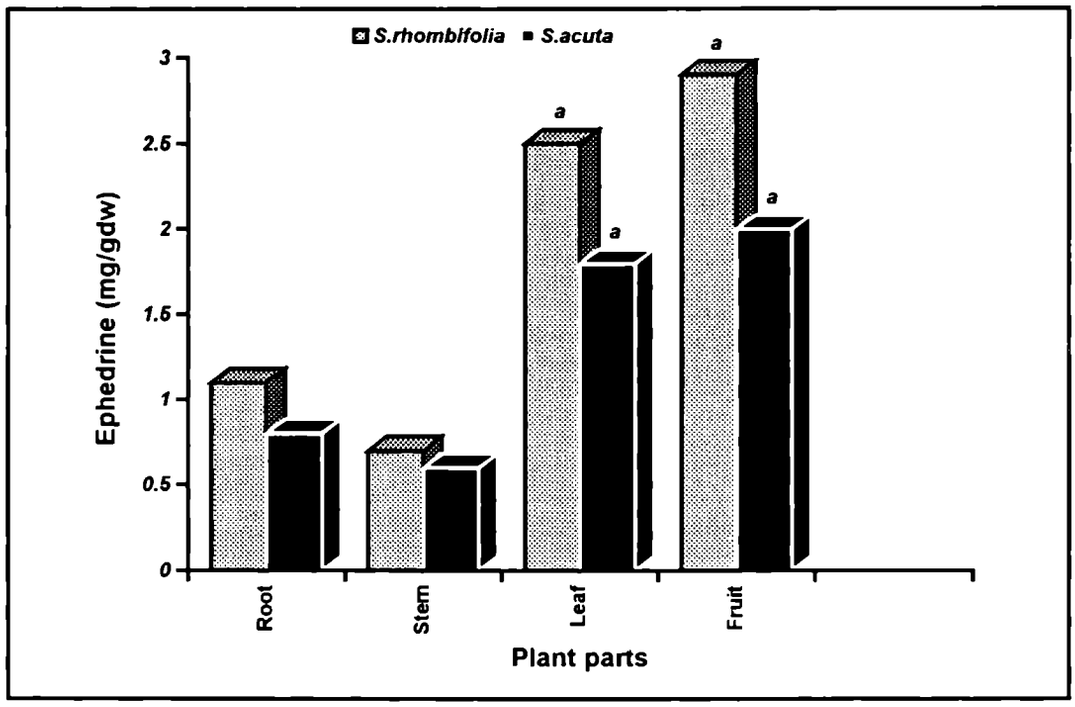
3.3.2 Selection of phytohormones

The explants of leaf segments, shoot tips, nodal segments, internodal segments and petioles were cultured on full MS medium supplemented with different concentrations of auxins and cytokinins. Among the different auxins and cytokinins tried, supplementation of the medium with 2.5 mg l^{-1}2,4-D alone or in combination

Plate 1 **Thin layer chromatogram of ephedrine (R_f value = 0.56)**
1. Plant extract; 2 & 4 – Pure ephedrine; 3 – Cell extract

Fig 3.1

Ephedrine content of different plant parts of *S.rhombifolia* and *S.acuta*



The data are the mean of 5 replicates. The bars marked with letter 'a' are statistically on par and significantly different from others. The cultures were incubated for 30 days at 25 ± 2^{0}C under light intensity of 1000 lux (16h photoperiod).

Plate 2 ***Sida rhombifolia*** **Linn. ssp.** ***retusa*** **(Linn.) Borssum plant**

with 1.0 mg l^{-1} BAP was selected as the best hormonal addenda for *S.rhombifolia*, whereas for *S.acuta* supplementation of the medium with 2.0 mg l^{-1} NAA and 0.5 mg l^{-1} kin was the best combination for callus initiation (Fig.3.2). For callus proliferation, while a lower concentration of 0.75 mg l^{-1} 2.4-D + 0.3 mg l^{-1} BAP was ideal for *S.rhombifolia*, 1.0 mg l^{-1} NAA + 0.2 mg l^{-1} kin was optimal for *S.acuta* (Fig.3.3).

3.3.3 Selection of explants

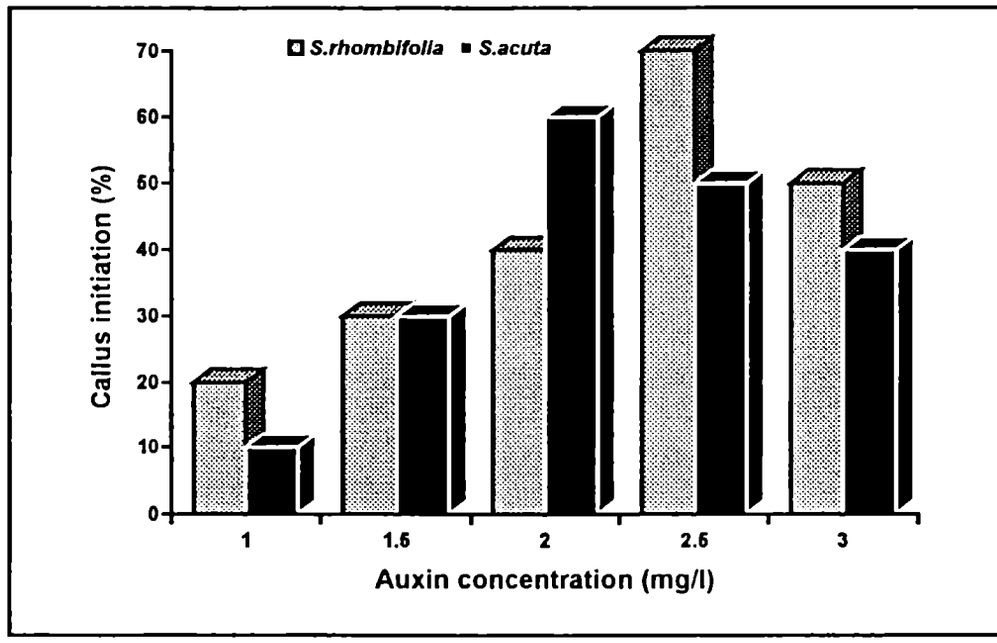
The explants of leaf segments, shoot tips, nodal segments, internodal segments and petioles were cultured on full MS medium supplemented with 2.5 mg l^{-1}2,4-D + 1.0 mg l^{-1} BAP for *S.rhombifolia* and with 2.0 mg l^{-1} NAA + 0.5 mg l^{-1} kin for *S.acuta*. In both the species, the leaf explants showed the maximum callus induction efficiency (Fig. 3.4). Further, the calli obtained from leaf explants of *S.rhombifolia* were friable and fast growing in nature. Whereas, in the case of *S.acuta*, the calli obtained from leaf explants were comparatively friable in spite of slow growing in nature. As our primary objective was to produce friable callus, to initiate cell suspension culture, the leaf of *S.rhombifolia* was selected as the best explant (Plate 3).

3.3.4 Ephedrine content of calli

The calli derived from the fruits of *S.rhombifolia* and *S.acuta* contained maximum ephedrine content (1.58 mg/gdw and 0.98 mg/gdw respectively followed by leaf calli (1.44 mg/g dw and 0.82 mg/gdw respectively). But the calli derived from the fruit explants were compact and slow growing in nature unlike the friable and fast

Fig 3.2

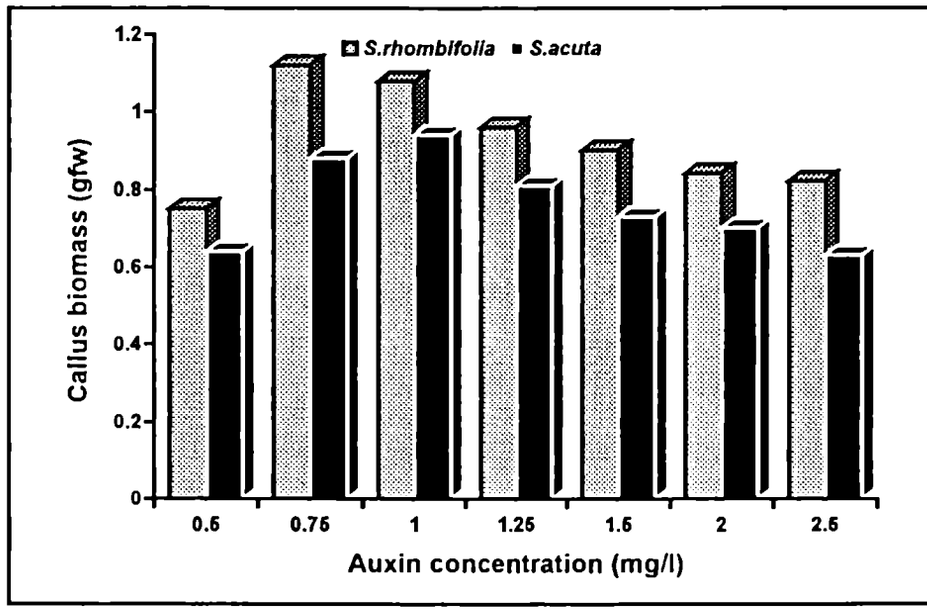
Effect of plant growth regulators on callus initiation of *S.rhombifolia* and *S.acuta*



For *S.rhombifolia* 2,4-D was used in combination with BAP (1mg/l) and for *S.acuta*, NAA was used in combination with kinetin (0.5mg/l). 300± 50mg callus was inoculated on 10ml MS medium(0.8% agar) and incubated for 21 days at 25±2^{0}C under 1000 lux light intensity (16h photoperiod). The data are the mean of 5 replicates.

Fig 3.3

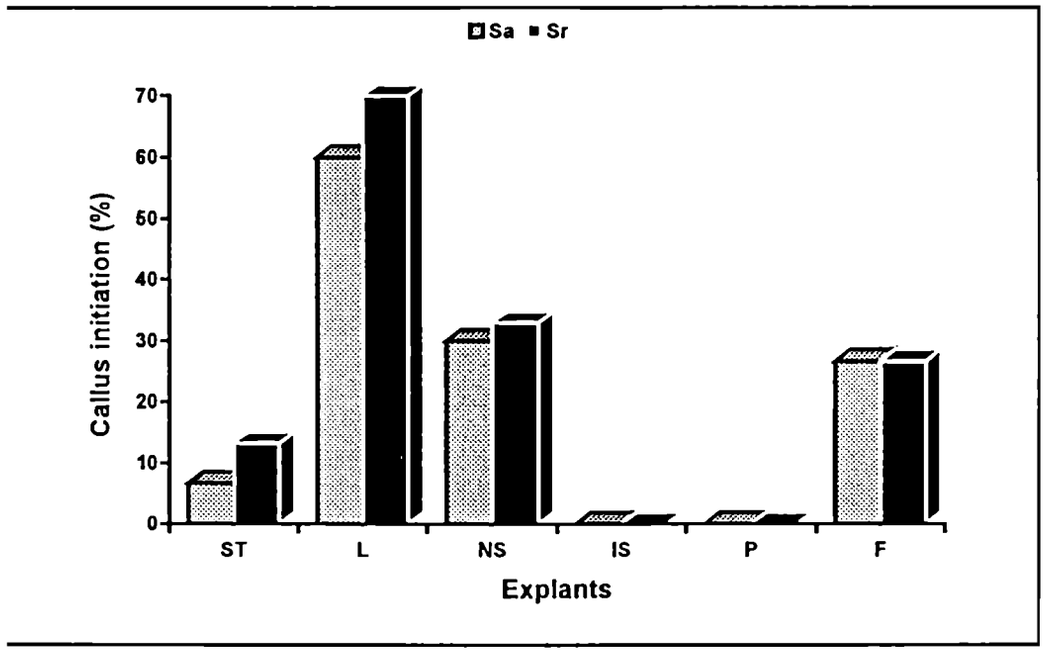
Effect of plant growth regulators on callus proliferation of *S.rhombifolia* and *S.acuta*



For *S.rhombifolia* 2,4-D was used in combination with BAP (0.3mg/l) and for *S.acuta*, NAA was used in combination with kinetin (0.2mg/l). 300± 50mg callus was inoculated on 10ml MS medium(0.8% agar) and incubated for 30 days at 25±2^{0}C under 1000 lux light intensity (16h photoperiod). The data are the mean of 5 replicates.

Fig 3.4

Callus induction efficiency of different plant parts of *S.rhombifolia* and *S. acuta*



- shoot tip, L-leaf, NS- nodal segment, IS- internodal segment, P-petiole and F-fr

S.acuta, the callus was initiated on MS medium supplemented with 2mg/l NAA a mg/l kin and in *S.rhombifolia* on MS medium with 2.5mg/l of 2,4-D and 1mg/l P. For each explant, 30 numbers were incubated at 25± 2^{0}C under a light intens 1000 lux (16h photoperiod).

growing nature of leaf calli (Plate 4). Further, the ephedrine content in fruit calli and leaf calli were on par statistically, in both the species (Fig.3.5).

3.4 Discussion

The physiological activity of different anxins vary in different genotypes (Endress, 1994). It was observed in the present investigation that the most ideal auxin: cytokinin requirement for callus initiation of *S.rhombifolia* and *S.acuta* was different. It might be due to the genotypic difference.

On subsequent subculturing, both the species showed, comparatively, requirement of lesser concentrations of auxin and cytokinin for further growth and proliferation. This smight be due to the fact that the synthetic auxins added to the medium might be absorbed onto the binding sites that are "unloaded" which would result in a high endogeneous auxin content (George & Sherrington, 1984). This high level of endogeneous auxin of the initiated calli might have favoured further proliferation of the tissues in the medium with low level of auxin: cytokinin content.

BAP was reported to be unsuitable for callus induction in *Sida* species (Sankar & Nair, 1997). Callus induction in *Sida cordifolia* was rapid in B5 medium supplemented with 2 mg l^{-1} NAA + 1.0 mg l^{-1} BAP (Nair *et al.* 1992). Whereas, in the present study, the most ideal auxin cytokinin combination for callus induction was 2.5 mg l^{-1} 2,4-D + 1.0 mg l^{-1} BAP for *S.rhombifolia* compared to 2.0 mg l^{-1} NAA + 0.5 mg l^{-1} kin for *S.acuta*. Hence it is inferred that a generalised phytohormonal

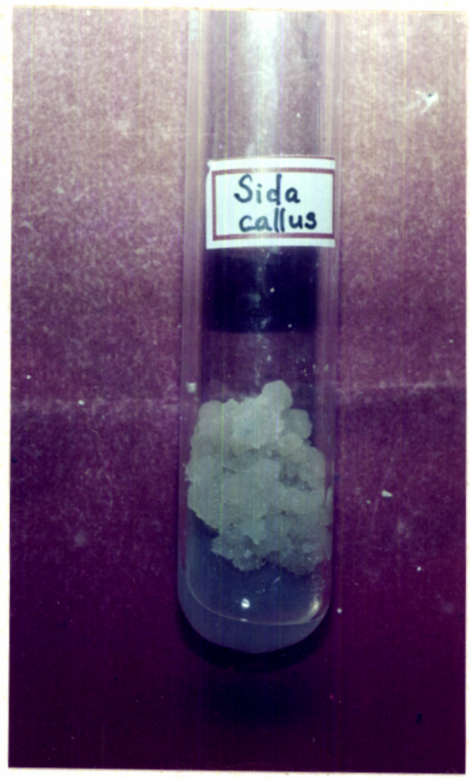


Plate 3 *Sida rhombifolia* callus

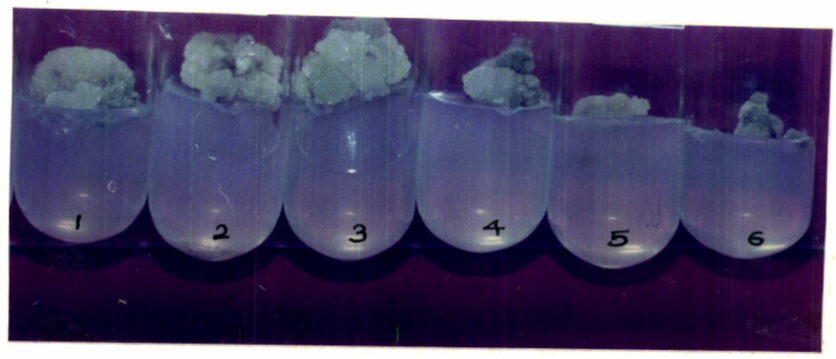
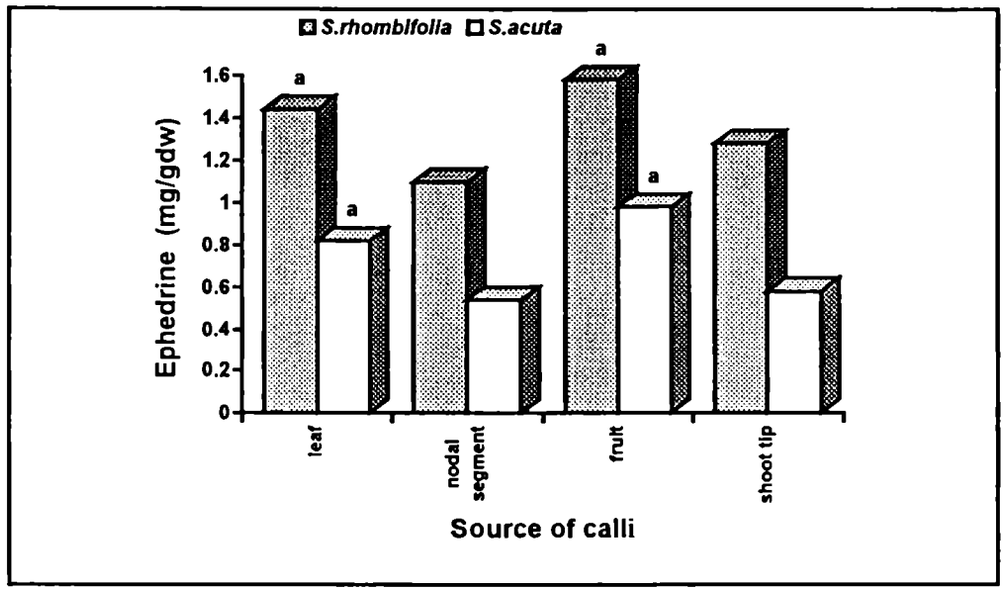


Plate 4 **Leaf and fruit calli of *S.rhombifolia***
1, 2 & 3 – friable and fast growing leaf calli
4, 5 & 6 – compact and slow growing fruit calli

Fig 3.5

Comparison of ephedrine content of calli derived from different explants of *S.rhombifolia* and *S.acuta*



The data represent mean of 5 replicates. The bars with letter 'a' are statistically on par and significantly different from others. The cultures were incubated for 30 days at 25 ± 2^{0}C, under a light intensity of 1000 lux (16h photoperiod).

combination for callus induction in *Sida* species is out of scope as it varies from species to species.

The callus tissues derived from various explants of *S.rhombifolia* and *S.acuta* varied considerably in their nature. The leaf explants of *S.rhombifolia* yielded friable and fast growing callus, ideally suited for suspension culture. Callus induction depends on the plant genotype, the source of origin of the explant and the physiological state of the tissue. Further, the nature of the callus tissue, its texture, compactness, friability and colouration also depends on the genotype, kind and age of the explant and even the season (Narayanaswamy, 1994).

Ephedrine content was less in the callus tissues of both the species studied, compared to their parent plant. As the secondary metabolite production is associated with the organisation of the tissues (Signs & Flores, 1990) and enhanced by stress factors (Rokem & Goldberg, 1985), the low alkaloid content in callus tissue might be attributed to the unorganised nature of the callus tissues. Further, the most ideal *in vitro* conditions which is highly suitable for the growth of the tissues might be unfavourable for secondary metabolite production. In the case of intact plants, the highly organised nature of the tissues and the chances for exposure to occasional stress conditions resulting from climatic fluctuations, pest and disease infestation etc. in the natural environment where it is grown, might be the factors favouring higher secondary metabolite content.

Considering the high callus induction efficiency, friable and fast growing nature of the callus and comparatively high alkaloid content, the leaf explant of *S.rhombifolia* was selected for detailed *in vitro* studies.

CHAPTER IV

Optimization studies on callus culture of *Sida rhombifolia* Linn. ssp. *retusa* (Linn.) Borssum

4.1 Introduction

The components of culture medium and cultural conditions have a profound effect on the rate of secondary metabolite production. In the present investigation, the media and the cultural conditions were optimized for the production of maximal biomass and ephedrine towards developing an ideal bioprocess for industrial production of this compound. For each experiment, the cultures (pH 5.6-5.8) were incubated for 30 days at 25±2°C, under a light intensity of 1000 lux (16h photoperiod) (unless otherwise stated).

4.2 Materials and methods

4.2.1 Source of plant material

The source of plant material was as described under section 3.2.2.

4.2.2 Callus induction and maintenance

The callus culture of *S.rhombifolia* was initiated and maintained as mentioned under section 3.2.3.

4.2.3 Growth measurements

The growth of callus culture was measured in terms of fresh weight and dry weight. Approximately 500 mg callus from the pooled fraction of fast growing callus lines was used as the inoculum. The exact initial weight of inoculum was determined from the

difference between the weight of culture tube before and after inoculation. After a specific period of incubation, the tube with the callus was weighed. After removing the callus aseptically, the tube with the medium was again weighed and the final weight of the callus was determined from the difference and expressed as gfw (gram fresh weight).

After determining the fresh weight the callus was dried in a hot air oven at 60°C to a constant weight (Approx. 24-48h) and the weight of cell biomass was expressed as gdw (gram dry weight).

4.2.4 Extraction procedure

The alkaloid was extracted from the callus as described under section 3.2.4.

4.2.5 Analysis of ephedrine content

The ephedrine content was analysed and estimated as mentioned under section 3.2.6.

4.2.6 Optimization of process parameters influencing biomass and ephedrine yield

Each process parameter was optimized independently for maximal biomass and ephedrine yield. After optimization of individual parameters independently, a time course experiment was carried out employing the process parameters at their optimized levels.

4.2.6.1 Temperature

The effect of incubation temperature on biomass production and ephedrine yield of *S.rhombifolia* callus culture was estimated by incubating the culture at various

temperatures (20, 25, 30 and 35°C). The callus biomass and ephedrine content were analysed as described previously under section 4.2.3 and 4.2.5 respectively.

4.2.6.2 Light intensity

The influence of light intensity on biomass production and ephedrine yield of *S.rhombifolia* was studied by incubating the culture at various light intensities (dark, 500, 1000 and 2000 lux). The biomass and ephedrine content were estimated as described under sections 4.2.3 and 4.2.5 respectively.

4.2.6.3 pH

The impact of pH of the medium on biomass production and ephedrine yield of *S.rhombifolia* callus culture was determined by subjecting the culture to various pH levels (5.0-7.0) adjusted in the medium using 1N HCl/1N NaOH.

4.2.6.4 Major salt formulations

The influence of major salt formulations in the medium on biomass and ephedrine yield of *S.rhombifolia* callus culture was determined by inoculating the culture in media containing SH (1972), N&N (1956), White's (1943), B5 (1968) & MS (1962) major salt formulations with MS micronutrients and vitamins. The biomass and ephedrine yield were estimated as detailed under sections 4.2.3 and 4.2.5 respectively.

4.2.6.5 Carbon sources

The influence of various carbon sources on biomass and ephedrine yield of *S.rhombifolia* callus culture was tested with sucrose, dextrose, maltose, lactose, galactose and xylose at 3% (w/v) level. The biomass and ephedrine yield were estimated as mentioned under sections 4.2.3 and 4.2.5 respectively.

4.2.6.6 Vitamin formulations

The impact of various vitamin formulations in the culture media on biomass and ephedrine yield of *S.rhombifolia* callus cultures was tested with N&N, SH, B5 and MS vitamin formulations. The biomass and ephedrine yield were analysed as detailed under sections 4.2.3 and 4.2.5 respectively.

4.2.6.7 Plant growth regulators

The impact of phytohormones on biomass and ephedrine production of *S.rhombifolia* callus culture was studied with various combinations of the auxins 2,4-D, NAA, IBA & IAA and the cytokinins BAP and Kinetin; incorporated into the basal MS medium. The biomass and ephedrine yield were estimated as described under sections 4.2.3 and 4.2.5 respectively.

4.2.6.8 Amino acids

The effect of various amino acids on biomass and ephedrine yield of *S.rhombifolia* callus culture was determined in MS basal medium incorporated with proline, glycine, L-

Aspartic acid, L-asparagin, L-cystine, L-tyrosine, L-glutamine, L-arginin and L-tryptophan at 2 mM level. All the amino acids were filter sterilized and used. The biomass and ephedrine yield of *S.rhombifolia* Linn. callus cultures in the MS basal medium with different combinations of aminoacids and with all the parameters at optimized level were estimated after incubating the cultures at 25±2°C under 1000 lux light intensity (16h. photoperiod) as detailed under sections 4.2.3 and 4.2.5 respectively.

4.2.7 Growth curve and production profile of callus cultures

The growth and production studies were carried out in the MS basal medium with all the optimized parameters. 500±50 mg callus tissue each was inoculated into the medium and incubated at 25±2°C under 1000 lux illumination (16h photoperiod) for a period of 36 days. Samples were harvested at regular intervals of 3 days and the biomass and ephedrine content were estimated as detailed under sections 4.2.3 and 4.2.5 respectively, and growth and production curve were plotted.

The specific growth rate (μ) was calculated using the formula (Ryu & Lee 1990; Madhusudhan *et al.* 1995).

$$\mu = \frac{\text{Log}_e N_t - \text{Log}_e N_o}{t}$$

where N_o - initial biomass

N_t - biomass after time 't'

e - base of natural logarithm (2.718)

The doubling time (td) was determined using the formula (Madhusudhan *et. al* 1995)

$$td = \frac{Log_e 2}{\mu} = \frac{0.693}{\mu}$$

where μ = specific growth rate.

4.3 Results

4.3.1 Influence of temperature

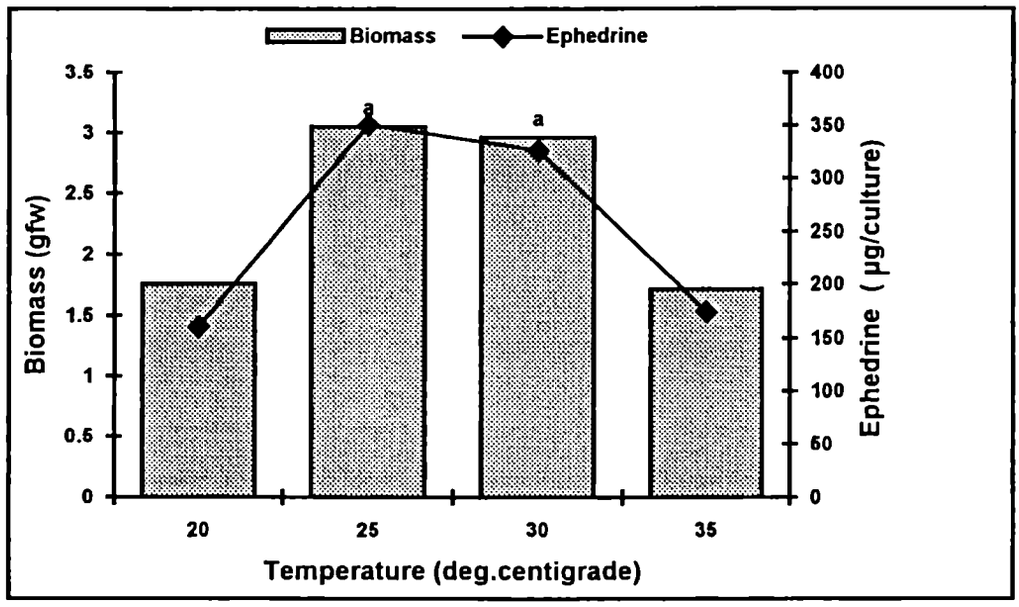
Results presented in Fig.4.1 indicate that the incubation temperature influenced the growth and ephedrine yield of *S.rhombifolia* callus cultures. Maximum yield of biomass (3.1 gfw) and alkaloid (350.1 µg/culture) was recorded at 25°C although the biomass and ephedrine yield at 25°C and 30°C were statistically on par.

4.3.2 Influence of light intensity

From the results depicted in Fig.4.2 it was inferred that illumination (16h photoperiod) at various light intensities and incubation in the dark did not influence biomass yield. Whereas, illumination at 2000 and 1000 lux (16h photoperiod) promoted ephedrine yield (456.5 µg/culture and 380.1 µg/culture respectively. However, it was observed that at 2000 lux light intensity, after two weeks of incubation, there was phenolic exudation which affected the growth adversely. Hence 1000 lux illumination was selected

Fig 4.1

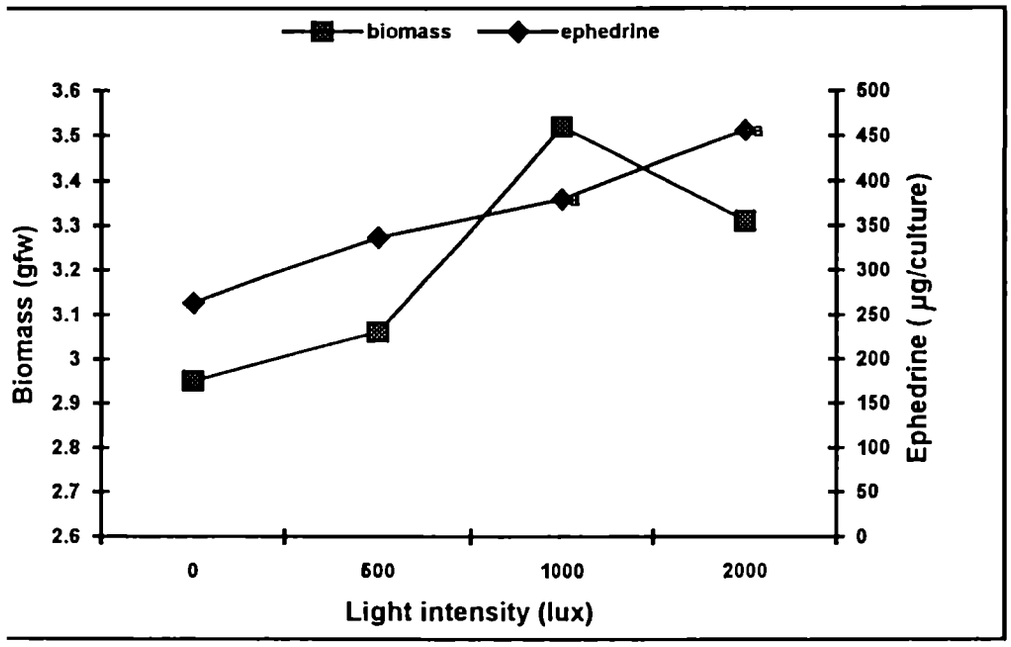
Impact of temperature on biomass and ephedrine yield of *S.rhombifolia* callus culture



Data represent average of 5 replicates. The bars/points marked with letter 'a' are statistically on par and significantly different from others. 500±50mg callus was inoculated on 10ml MS medium with 0.8% agar and 2,4-D (2.5mg/l) + BAP.(1mg/l) Incubation 30 days, at 25±2^{0}C under 1000 lux light intensity (16h photoperiod).

Fig 4.2

Influence of light intensity on growth and ephedrine yield of *S.rhombifolia* callus culture



data represent average of 5 replicates.The points with letter 'a' are statistically
and significantly different from others. 500±50mg callus tissue was inoculated
l MS medium (0.8% agar) supplemented with 2,4-D (2.5mg/l) & BAP(1.0m
incubated for 30 days under 1000 lux light intensity (16 h photoperiod) at 25±2
5.8.

as optimal illumination taking into consideration, the maximal biomass (3.5 gfw) and ephedrine yield (380.1 µg/culture).

4.3.3 Influence of pH

The pH of the culture medium influenced the biomass and ephedrine yield of *S.rhombifolia* callus cultures (Fig.4.3). The maximum biomass (3.1 gfw) and ephedrine yield (332.5 µg/culture) were recorded at pH 5.8. Nevertheless, the biomass and alkaloid yields were statistically on par over a pH range of 5.6-6.2.

4.3.4 Effect of major salt formulations

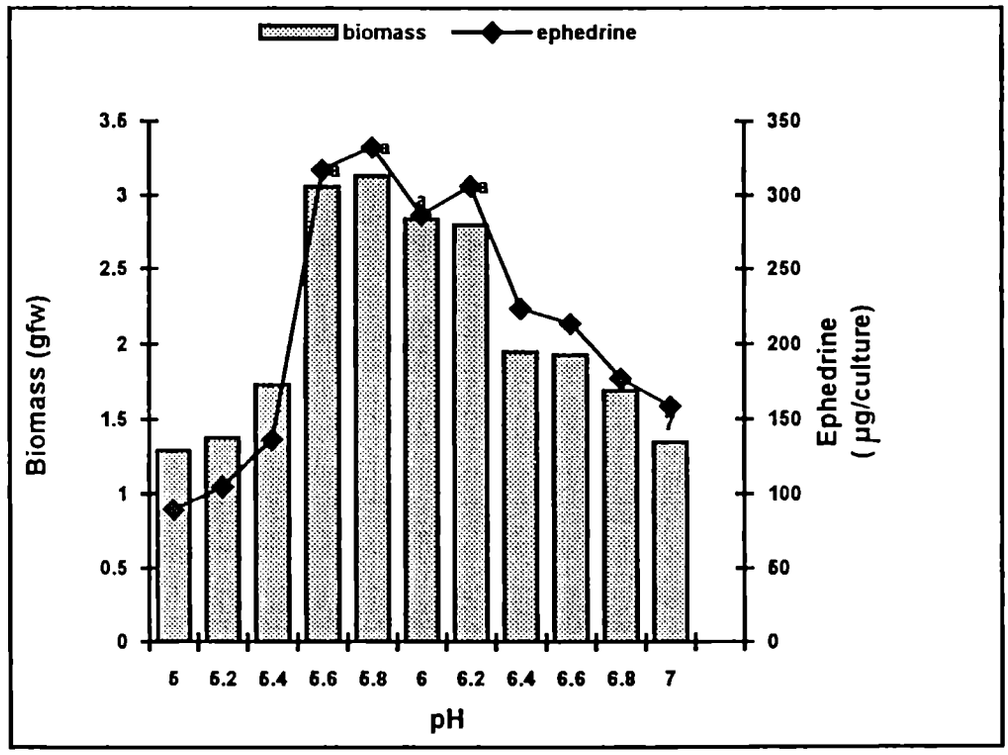
Among the various major salt formulations tried, the MS major salt formulation promoted maximal growth (3.6 gfw) and alkaloid production (323.8 µg/culture) followed by B5 and SH major salt formulations (Fig.4.4).

4.3.5 Influence of carbon sources

The effect of different concentrations of sucrose on growth and tissue response of *S.rhombifolia* callus was studied. The results presented in fig.4.5 indicated that the optimum concentration of sucrose for better growth was 3%. Further increase in concentration resulted in reduction of growth. It was also observed that, the tissues retained normal colour and texture with sucrose levels upto 3% and with further increase in concentration (4-6%), the tissues turned yellowish brown and the texture was changed from friable to compact. Hence 3% level of sucrose was selected as ideal concentration for further studies.

Fig 4.3

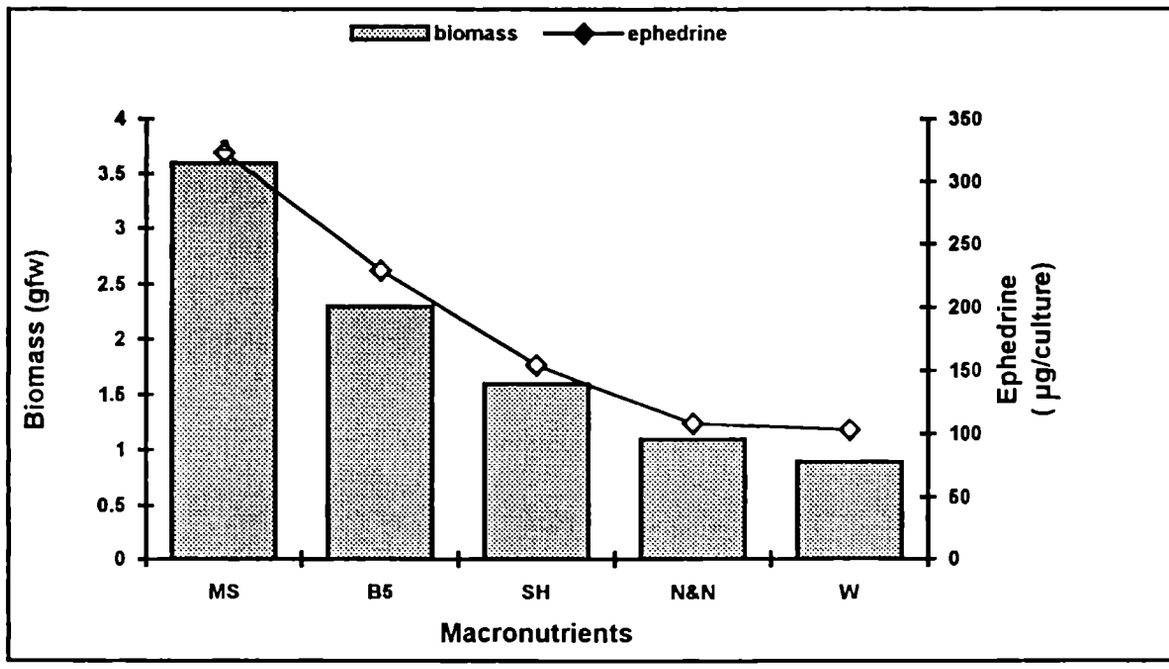
Influence of pH on growth and ephedrine yield of *S.rhombifolia* callus culture



The data represent average of 5 replicates.The bars/points with letter 'a' are statistically on par and significantly different from others.500±50mg callus tissue was inoculated on 10ml MS medium (0.8% agar) supplemented with 2,4-D (2.5mg/l) & BAP(1.0mg/l) and incubated for 30 days under 1000 lux light intensity (16 h photoperiod) at 25±2^{0}C.

Fig 4.4

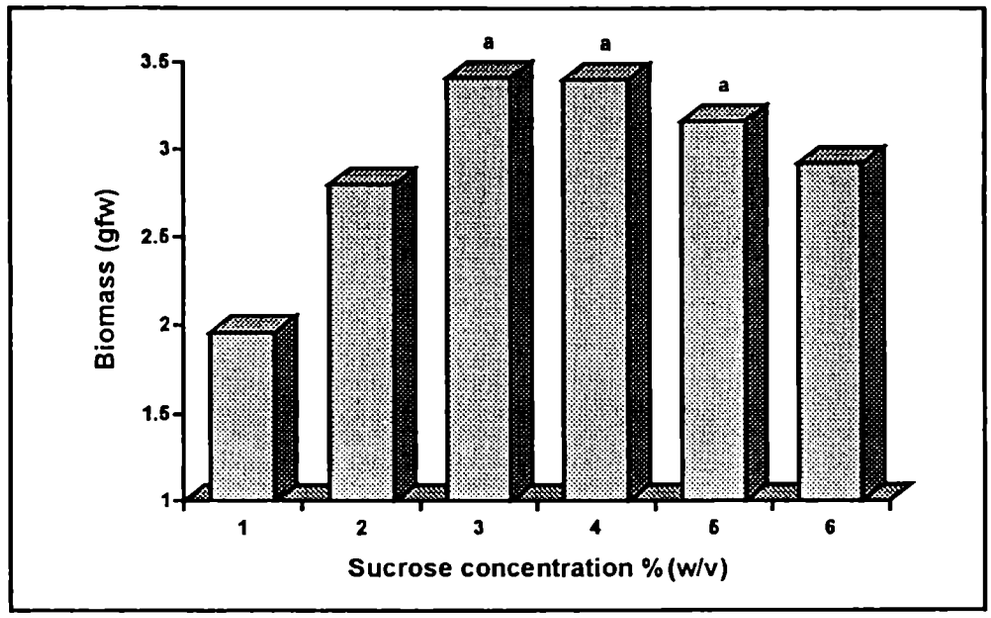
Effect of macronutrients on biomass and ephedrine yield of *S..rhombifolia* callus culture



MS-Murashige &skoog, B5- Gamborg's B5, SH- schlieden & Hildebrandt, N&N- Nitsch & Nitsch and W- white's. The data represent average of 5 replicates.The bar/point with letter 'a' is significantly different from others. 500±50mg callus tissue was inoculated on 10ml MS medium (0.8% agar) supplemented with 2,4-D (2.5mg/l) & BAP (1.0mg/l) and incubated for 30 days under 1000 lux light intensity (16 h photoperiod) at 25±2^{0}C; pH -5.8.

Fig 4.5

The impact of different concentrations of sucrose on growth of *S.rhombifolia* callus culture



The data represent average of 5 replicates.The bars with letter 'a' are statistically on par and significantly different from others. 500±50mg callus tissue was inoculated on 10ml MS medium (0.8% agar) supplemented with 2.5mg/l of 2,4-D & 1.0 mg/l of BAP and incubated for 30 days under 1000 lux light intensity (16 h photoperiod) at 25±2^{0}C; pH -5.8.

Among the six carbon sources tried (Fig.4.6), sucrose favoured maximal growth (3.3 gfw) and ephedrine yield (379.2 µg/culture) followed by dextrose and maltose. Lactose, galactose and xylose all inhibited callus growth and yielded only 30-40% of ephedrine compared to that obtained at 3% sucrose level.

4.3.6 Influence of vitamin formulations

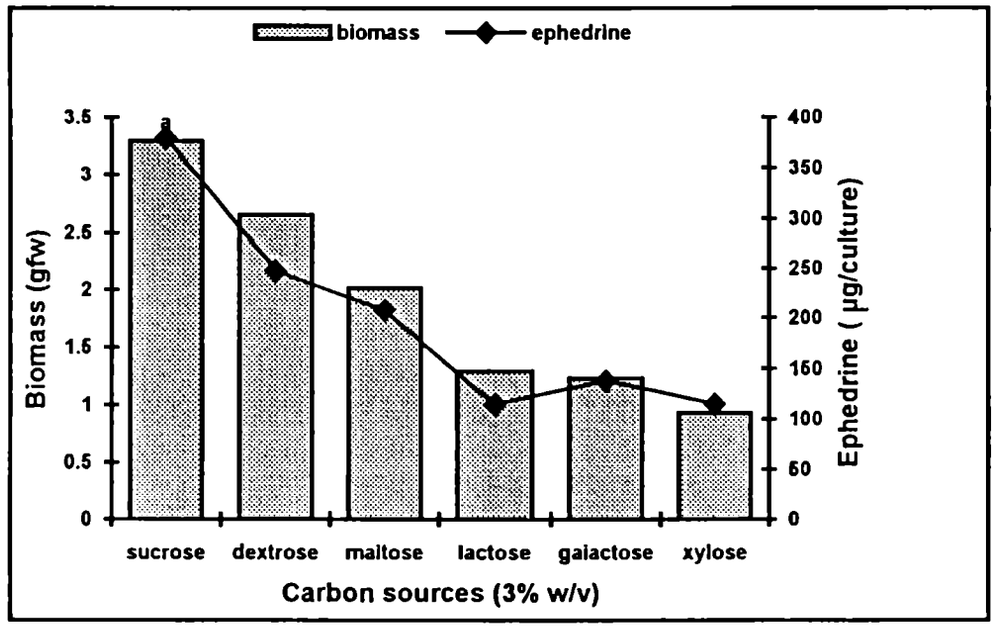
From the results presented in fig.4.8 it is evident that combinations of vitamin formulations favoured maximal biomass and ephedrine yield compared to individual additions. Thus a combination of (MS+B5) supported maximal biomass (3.4 gfw) and ephedrine yield (367.2 µg/culture) compared to MS alone which yielded 3.0 gfw biomass and 337.9 µg/culture ephedrine. With respect to ephedrine yield, MS, MS+B5 and MS+N&N were statistically on par indicating the enhancing effect of MS vitamin formulation either alone or in combination in the medium.

4.3.7 Influence of plant growth regulators

Results presented in Table 4.1 show the strong impact of plant growth regulators on growth and alkaloid production of *S.rhombifolia*. Among the various combinations tested, combination A1 (2.5 mg l^{-1}2,4-D + 1.0 mg l^{-1} BAP) resulted in maximum biomass (3.4 gfw) and alkaloid production (411.8 µg/culture). Nevertheless, combinations B1 (2.5 mg l^{-1} 2,4-D+1.0 mgl-1 kin), C1 (5.0 mg l^{-1}2,4-D+1.0 mg l^{-1} BAP) and C3 (5.0 mg l^{-1}

Fig 4.6

Impact of carbon sources on biomass and ephedrine production of *S.rhombifolia* callus culture



Data represent average of 5 replicates. The bar/point marked with letter 'a' is significantly different from others. 500±50mg callus was inoculated on 10ml MS medium with 0.8% agar and 2,4-D (2.5mg/l) & BAP.(1mg/l). Incubation period 30 days at 25±2°C under 1000 lux light intensity (16h photoperiod).

Table 4.1

Influence of plant growth regulators on biomass and ephedrine yield of *S.rhombifolia* Linn. callus culture

Plant growth regulator combination (mgl^{-1})	Biomass gfw	Ephedrine µg/culture
A1 2,4-D (2.5) + BAP (1.0)	3.38 a	411.8 a
A2 NAA (2.5) + do	2.15	301.0
A3 IBA (2.5) + do	1.94	284.8
A4 IAA (2.5) + do	2.15	239.8
B1 2,4-D (2.5) + Kin (1.0)	2.93 a	379.1 a
B2 NAA (2.5) + do	2.18	328.5
B3 IBA (2.5) + do	1.86	299.3
B4 IAA (2.5) + do	1.97	226.0
C1 2,4-D (5.0) + BAP (1.0)	2.91 a	337.9 a
C2 NAA (5.0) + do	2.41	307.0
C3 IBA (5.0) + do	2.32	322.7 a
C4 IAA (5.0) + do	1.86	165.9
LSD (0.05)	0.49	89.7

LSD – Least Significant Difference. The data represent mean of 5 replicates. The figures with letter 'a' are statistically on par and are significantly different from others. 500±50 mg callus was inoculated on 10 ml solid MS medium and incubated for 30 days under 1000 lux light intensity (16h photoperiod) at 25 ±2°C: pH of the medium 5.8.

1BA + 1 mg l^{-1} BAP) were also statistically on par with combination A1 with respect to alkaloid production.

4.3.8 Influence of amino acids

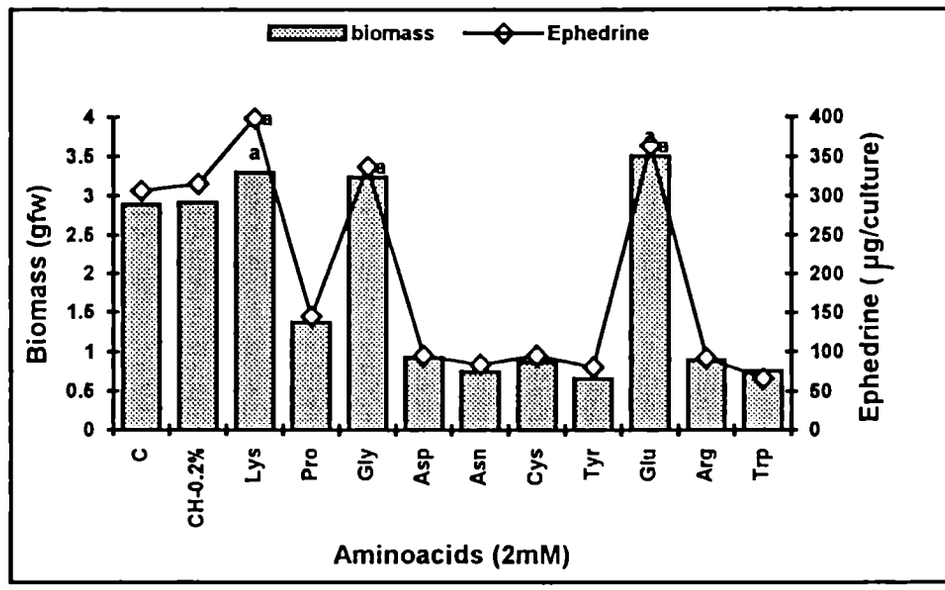
Among the amino acids tested (Fig.4.7), the media supplemented with L-lysine and L-glutamine enhanced significantly the biomass (3.3 and 3.2 gfw respectively) and ephedrine yield (398.4 and 337.3 µg/culture respectively), compared to others. After optimization of various culture media parameters, the MS macronutrients and micronutrients, (MS+B5) vitamin combinations and (2.5 mg l^{-1}2,4-D + 1.0 mg l^{-1}BAP) phytohormonal combinations were selected as most ideal for callus culture growth and ephedrine yield of *S.rhombifolia* Linn. Four different media were formulated with different amino acid contents (MSSR-1 with 2mM L.glutamine, MSSR-2 with 2 mM glycine, MSSR-3 with 2mM L-lysine and MSSR-4 with 1 mM each of L-glutamine, glycine and L-lysine) and biomass and ephedrine yield were analysed. From the results presented in table 4.2, it was observed that MSSR-4 medium yielded maximum biomass (4.23 gfw) and ephedrine (492.2 µg/culture), though statistically on par with MSSR-1 and MSSR-3. The optimized medium, MSSR-4, yielded 46% increase in biomass yield and 32% increase in ephedrine yield compared to callus culture of *S.rhombifolia* Linn. in basal MS medium alone.

4.3.9 Growth pattern and production profile of alkaloid

Data presented in fig.4.9, for the growth analysis of *S.rhombifolia*, indicated that the exponential growth phase is of 14 days after an initial 7 days of lag phase. This is

Fig 4.7

Influence of aminoacids on biomass and ephedrine production of *S.rhombifolia* callus culture



C- Control, CH- casein hydrolysate, Lys- L-Lysine, Pro- Proline, Gly- Glycine, Asp- L-Aspartic acid, Asn- L-Asparagin, Cys- L-Cysteine, Tyr- L-Tyrosine, Gln- L-Glutamin, Arg- L-Arginin, Trp- L.Tryptophan.

Data represent average of 5 replicates. The bars/points marked with letter 'a' are statistically on par and significantly different from others. 500±50mg callus was inoculated on 10ml MS medium with 0.8% agar and 2.5mg/l of 2,4-D & 1mg/l of BAP. Incubation period 30 days at 25±2°C under 1000 lux light intensity (16h photoperiod).pH 5.8.

Table 4.2

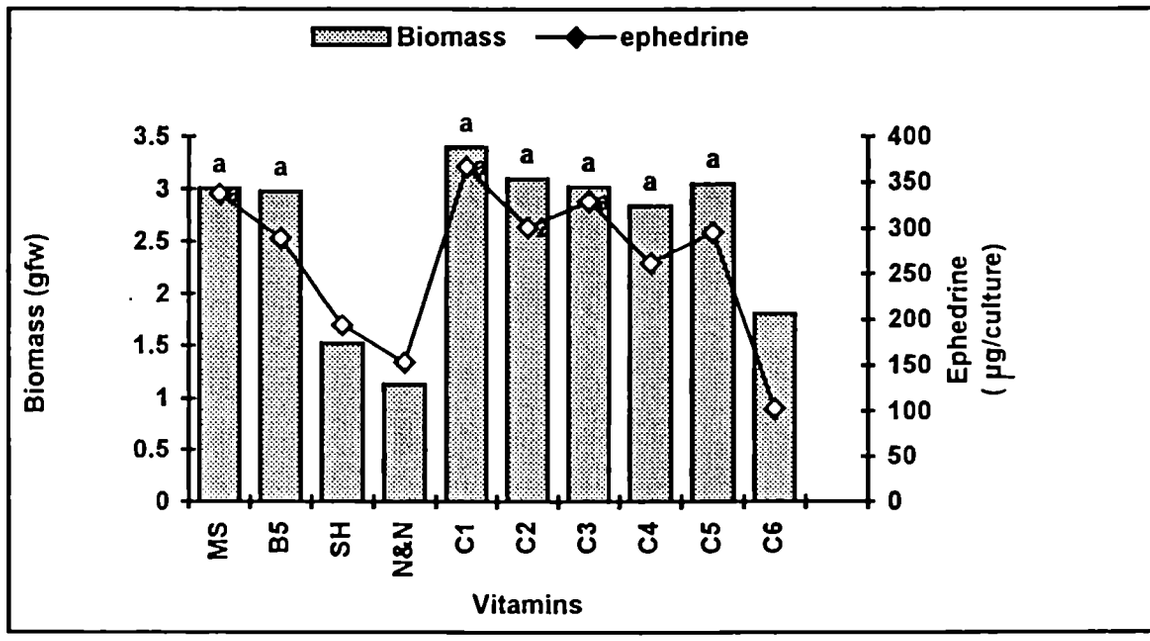
Impact of the selected aminoacids on ephedrine yield of *S.rhombifolia* callus culture

Media composition	MS	MSSR-1	MSSR-2	MSSR-3	MSSR-4
Macronutrients	MS	MS	MS	MS	MS
Micronutrients	MS	MS	MS	MS	MS
Vitamins	MS	($MS+N_5$)	($MS+N_5$)	($MS+N_5$)	($MS+N_5$)
Aminoacids (mM)	MS	GLN(2)	Gly(2)	LYS(2)	LYS-1 GLY-1 GLY-1
Phytohormones					
2,4-D(mg l^{-1})	2.5	2.5	2.5	2.5	2.5
BAP(mg l^{-1})	1.0	1.0	1.0	1.0	1.0
Biomass (gfw)	3.2	3.95 a	3.65 a	4.16 a	4.23 a
Ephedrine (µg/culture)	337.8	406.4 a	393.2	467.5 a	492.2 a

The data represents mean of 4 replicates. The figures with letter 'a' are statistically on par and are significantly different from others. 500±50 mg callus was inoculated on 10 ml solid medium and incubated for 30 days under 1000 lux light intensity (16h photoperiod) at 25 ±2°C: pH of the medium 5.8.

Fig 4.8

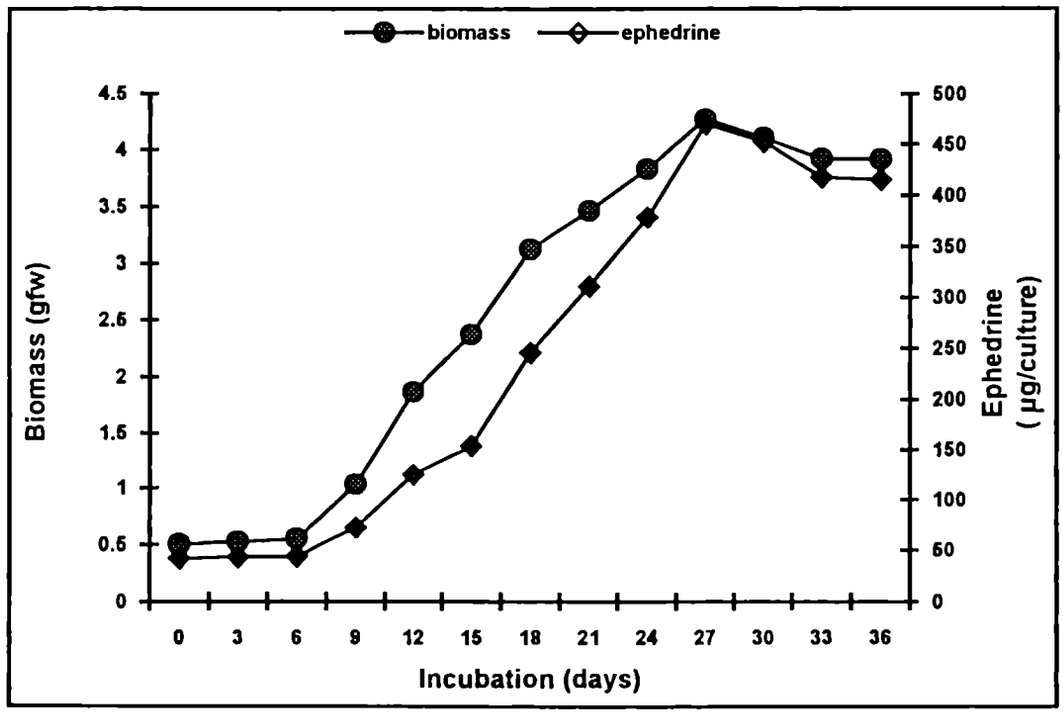
Influence of vitamins on biomass and ephedrine yield of *S.rhombifolia* callus culture



MS-Murashige &skoog, B5- Gamborg's B5, SH- schlieden & Hildebrandt, N&N-Nitsch & Nitsch, C1-MS+B5, C2- MS+SH, C3-MS+N&N, C4- B5+SH, C5-B5+N&N and C6-SH+N&N. The data represent average of 5 replicates.The bars/points with letter 'a' are statistically on par and significantly different from others. 500±50mg callus tissue was inoculated on 10ml MS medium (0.8% agar) supplemented with 2,4-D (2.5mg/l) & BAP (1.0 mg/l) and incubated for 30 days under 1000 lux light intensity (16 h photoperiod) at 25±2^{0}C; pH-5.8.

Fig 4.9

Time course experiment on growth and ephedrine production in *S.rhombifolia* callus culture



The data represent average of 5 replicates. 500±50mg callus tissue was inoculated on 10ml MS medium (0.8% agar) supplemented with 2,4-D (2.5mg/l) & BAP (1.0 mg/l) and incubated for 30 days under 1000 lux light intensity (16 h photoperiod) at 25±2^{0}C; pH-5.8.

followed by a progressive deceleration stage of 7 days before the setting in of stationary phase of growth. Ephedrine production in *S.rhombifolia* (Fig.4.9) was growth associated although the maximum production (469.7 µg/culture) was on 27th day of culture. Specific growth rate (µ)of 0.132/day (the biomass increased by about 13.2% in one day during the exponential growth phase) and doubling time (td) of 5.25 days in callus cultures were observed during time course experiment.

4.4 Discussion

The biomass production and ephedrine yield were significantly influenced by the cultural conditions. The optimum temperature that promoted the maximum biomass and alkaloid yield was 25°C, though these were statistically on par over a temperature range of 25-30°C. Illumination (16h photoperiod) at various light intensities and incubation in the dark did not show any significant influence on biomass yield, while illumination at 1000-2000 lux light intensity promoted significantly high ephedrine yield. But at 2000 lux light intensity, phenolic exudation and consequently the browming of tissues and reduced growth were observed in the later stages of culture, though the alkaloid production was high. Hence in this investigation, 1000 lux light intensity was preferred in the subsequent experiments.

The cultural conditions, particularly light regime and temperature influence the general growth and metabolism of tissues in cultures, and consequent production of

secondary metabolites (Narayanaswamy, 1994). It was reported that light stimulated the production of alkaloids in *Ephedra gerardiana* callus cultures (Ramawat & Arya, 1979a). In order to ensure the availability of the essential elements and the plant growth regulators provided in the medium to the plant tissue, the suitable pH has to be optimized for each and every system. Both the biomass and alkaloid yield were statistically on par over a pH range of 5.6-6.2 though the optimum pH of the medium that promoted the maximal biomass and alkaloid yield was 5.8.

The most important step in deriving a plant tissue culture medium for a particular system is the selection of macronutrient ions at appropriate concentration and balance. Gamborg's B5 medium was reported as the best for callus initiation and growth of a related species *Sida cordifolia* (Nair *et. al* 1992) while half concentration of MS medium was reported as ideal for callus initiation in *Sida* spp. (Sankar & Nair, 1997).

Whereas in the present investigation, full MS medium was ideal for callus initiation and growth of *S.rhombifolia* Linn. The cultures in MS medium provided 36% increase in biomass yield and 29% increase in ephedrine yield compared to Gamborg's B5 medium. The exact reason for the variability of growth in different basal media is not clear. The formulations of one medium differs from the other in macronutrients, micronutrients and even in organic supplements like vitamins and sugars (Anderson *et. al* 1987). There is variation in the ratio of NH_4^+ and NO_3^- among the four media used in the present study (Table 4.3). The concentration of NH_4^+ is very low in B5 medium compared

Table 4.3

Concentration of macronutrient ions in the five media

Media	Concentration in milliequivalents							
	NO_3-	H_2PO_4	SO_4-	K^+	Ca^{++}	Mg^{++}	NH_4^+	Total
MS	39.4	1.3	3.0	20.0	6.0	3.0	20.6	60.0
B5	24.7	1.1	4.0	24.7	2.0	2.0	2.0	26.8
SH	24.7	2.6	3.2	24.7	2.7	3.2	2.6	27.3
White	3.3	0.1	8.7	1.7	2.5	5.8	--	3.3
N&N	19.8	1.8	2.0	39.9	0.3	2.0	--	19.8

MS – Murashige & Skoog (1962), B5 – Gamborg *et al.* (1968), SH – Schenk & Hildebrandt (1972), White – White (1954) and N&N – Nitsch & Nitsch (1956).

to MS. But the presence of inorganic nitrogen in the form of both NH_4 and NO_3 in the MS medium may not be the sole reason for the variation in the growth of callus tissue in these media.

Carbohydrate nutrition is important in the growth of plant tissues grown in culture. Ramawat & Arya (1977) reported sucrose as the best carbon source for *E.gerardiana* and *E.foliata*. In this study also, sucrose was the best carbon source followed by dextrose and maltose. Sucrose at 3% (w/v) level yielded around 20% increase in biomass and about 35% increase in alkaloid yield, compared to dextrose. Moreover when the concentration of the sucrose was increased, there was a reduction in growth rate, change in the colour and texture of callus.

Vitamins play a catalytic role in cell metabolism apart from being a factor in accessory food supply but their requirements vary from species to species (Narayanaswamy, 1994). Use of (MS+B5) vitamin combination yielded around 11.5% increase in biomass and 8% increase in alkaloid yield, compared to the use of MS vitamins alone. The presence of higher concentration of thiamine hydrochloride in B5 vitamins might be one of the factors which effected positive response (Table 4.4).

Sankar and Nair (1997) reported that (1 mg l^{-1} NAA + 0.5 mg l^{-1} kin) was ideal for initiating callus culture of *Sida* species and BAP was unsuitable for callus initiation and growth. But in this investigation, BAP favoured both initiation and growth of *S.rhombifolia* callus. Further, the present results are in agreement with Nair *et. al* (1992)

Table 4.4

Vitamin combinations of different media

Vitamins	N&N mg l^{-1}	SH mg l^{-1}	MS mg l^{-1}	B_5 mg l^{-1}
Inositol	100	1000	100	100
Nicotinic acid	5.0	5.0	0.5	1.0
Pyridoxin HCl	0.5	0.5	0.5	1.0
Thiamine HCl	0.5	5.0	0.1	10.0
Folic acid	0.5	--	--	--
Biotin	0.05	--	--	--
Glycine	2.00	--	2.00	2.00

who reported the favourable effect of BAP in callus culture initiation of a related species *Sida cordifolia*.

Addition of amino acids also supported both the biomass and ephedrine yeild. Addition of 2 mM level of L-glutamine, L-lysine and glycine stimulated biomass yield by 18.0%, 12.4% and 10.8% respectively. Similarly addition of L-lysine resulted in an increase of 23% alkaloid yield and 15.7% by L-glutamine. These amino acids might be used for growth and ephedrine biosynthesis in the later stages of growth when the auxin and nitrogen were exhausted in the medium.

Although all the four media with different amino acid combinations yielded high biomass, significantly high alkaloid yield was promoted by MSSR-1, MSSR-3 and MSSR-4 in which 2 mM glutamine, 2 mM lysine and 1 mM each of glutamine, lysine and glycine respectively were added apart from MS macro and micronutrients, (MS + B5) vitamins supplemented with (2.5 mg l^{-1}2,4-D + 1.0 mg l^{-1} kin). Among the 4 combinations, MSSR-4 yielded maximal biomass and alkaloid.

In the present investigation, the maximum growth and production of *S.rhombifolia* was observed after 27 days of incubation. Nair *et. al* (1992) have reported the maximum growth of S.cordifolia after 40 days of incubation in B5 medium and 30 days in MS medium. No other reports were available with respect to the growth and production profile of *S.rhombifolia* callus cultures for comparison purpose. The maximum

production of alkaloid was also recorded on 27th day and the highest titre remained stationary for about a week during the stationary phase.

Based on the results of optimization, MSSR-4 medium was optimized for *in vitro* culture of *S.rhombifolia* in which the major difference from MS basal medium was the addition of (MS+B5) vitamin mixture and 1 mM each of lysine, glutamine and glycine supplemented with 2.5 mg l^{-1} 2,4-D + 1.0 mg l^{-1} BAP.

CHAPTER V

Studies on suspension culture of *Sida rhombifolia* Linn. ssp. *retusa* (Linn.) Borssum

5.1 Introduction

The production of mass amounts of plant tissue by submerged culture (Tulecke & Nickell, 1959) and its potential use for the study of biosynthesis of secondary compounds such as alkaloids and steroids have been suggested (Nickell & Tulecke, 1960).

Cell suspension cultures offer the possibility to establish nearly homogeneous culture conditions for all cells. Nevertheless, individual cells often differ considerably in their secondary metabilite content. Suspension cultures are initiated by transfer of the most friable sectors of an established callus tissue into an agitated liquid medium. Cells are freed from the parent callus and dispersed in the liquid medium where they divide to form small cell aggregates. They are maintained by serial subculture under the right conditions of agitation. After several passages, the friability of the culture increases. A suspension culture consists of cells and cell aggregates dispersed in liquid medium and actively growing under agitation and aeration. In this chapter, the initiation of suspension culture of *S-rhombifolia* Linn and the growth and production pattern were studied.

5.2 Materials and methods

5.2.1 Source of plant material

The source of plant material was as described under section 3.2.2.

5.2.2 Callus induction and maintenance

The callus culture of *S.rhombifolia* was initiated and maintained as described under section 3.2.3.

5.2.3 Initiation and maintenance of suspension culture

Callus tissue (approx. 4g) was transferred to 40 ml of liquid MS medium (2.5 mg l^{-1} of 2,4-D + 1 mg l^{-1} of BAP) in 150 ml Erlenmeyer flasks. They were incubated for 2 weeks on a rotary shaker (Orbitek, India) at 90 rpm under light intensity of 1000 lux for 16 h photoperiod. The cell suspensions were filtered through sieves (850 μm) to obtain single cells and few celled aggregates for use as inoculum for subsequent subculturing. Fine cell suspension culture was obtained by repeated subculturing of callus and removing small clumps at every stage. The cell suspension culture was maintained by subculturing at 2 weeks interval.

5.2.4 Detection and identification of the contaminants

The contaminant was detected by sterility test as described by Cassels (1986). Sap from the surface sterilized leaf explants of *S.rhombifolia* was plated on two different solidified sterility test media (Nutrient agar & Potato Dextrose Agar, Hl media, India) and incubated at 30°C for 48h. and assessed for the growth of contaminants.

Isolates obtained as contaminants were identified based on their morphological, physiological and biochemical properties (Claus & Berkeley, 1986).

5.2.5 Antibiotic sensitivity test

Antibiotic sensitivity tests for the contaminants were carried out employing disk diffusion test using antimicrobial octodiscs (OD 002 GII plus and OD 005 GI Minus from Hl media, India). Nutrient agar plates were inoculated with the log phase broth culture employing spread plate technique and the antibiotic octodiscs were placed gently over it using a sterile forceps and pressed gently. The petriplates were then incubated at 30°C for 18-24h. Antibiotic sensitivity was assessed by measuring the diameter of the clear zone around the colonies developed on the plates.

5.2.6 Dilution susceptibility test

Based on the results of antibiotic sensitivity test, the antibiotics which expressed maximum sensitivity were selected, filter sterilized using millipore filter sterilizer assembly and tested for Minimal Inhibition Concentration (MIC). The two most sensitive antibiotics viz., gentamycin and ciprofloxacin were incorporated at a concentration, varying from 2 $\mu g\ ml^{-1}$ to 20 $\mu g\ ml^{-1}$, in 10 ml of nutrient broth, inoculated with the contaminant bacteria (1% v/v) at a conc. of 0.1 OD and determined the turbidity at 660 nm after 20h of incubation at 30°C. The minimum concentration at which growth of the contaminant bacteria was completely inhibited was selected as the MIC of each selected antibiotics. The

MLC (Minimal Lethal Concentration) was ascertained by subculturing the tubes showing no growth into fresh medium lacking antibiotics. The lowest antibiotic concentration from which the micro organisms do not recover and grow when transferred to fresh medium was selected as the MLC.

5.2.7 Control of the contaminant bacteria

The selected antibiotics were filter sterilized using millipore filter sterilizer assembly. The shake flask cultures of *S.rhombifolia* were added with different concentrations of selected antibiotics and incubated at 25±2°C in an orbital shaker (90 rpm) for two weeks. The plant cell density and bacterial count (number/ml) were observed using a haemocytometer under Phase Contrast Microscope and the plant cell viabiloity was determined by dye exclusion procedure for viable cells using Evan's blue (Gaff & Okong'O-Ogola, 1971).

5.2.8 Growth measurements

The growth of suspension cultures was measured in terms of wet weight and dry weight of cells. To determine the fresh cell weight, the cell suspension was filtered through a preweighed whatman No.1 (wet condition) filter paper on a buchner funnel under slight vacuum. The cells were washed with distilled water and drained fully under vacuum, reweighed the cells with filter paper and expressed the weight of cell biomass as g wet cell weight l^{-1}.

To determine the dry cell weight, the cell suspension was filtered through a preweighed whatman No.1 filter paper on a buchner funnel under slight vacuum.

The cells were washed with distilled water and drained fully under vacuum. Dried the cells with filter paper in a hot air oven at 60°C to a constant weight (approx. 12-24h), reweighed the cells with filter and expressed the weight of cell biomass as g dry cell weight l^{-1}.

5.2.9 Extraction procedure

a) From the cells

The cells were separated from the suspension by centrifugation (Kubota, Japan), at 10000 rpm for 10 minutes and washed with distilled water thrice and drained under vacuum. The alkaloid was extracted from the cells as described under section 3.2.4.

b) From the medium

Ephedrine from the culture medium was extracted into (3:1) ether: chloroform mixture (3x10 ml), rendered strongly alkaline with ammonia, and the organic fractions were pooled and used for assay.

5.2.10 Analysis of ephedrine content

The ephedrine contents of both the cells and medium were analysed as detailed under section 3.2.6.

5.2.11 Influence of pH

The impact of pH on biomass production and ephedrine yield of *S.rhombifolia* Linn. suspension culture was determined by subjecting the cultures

to various pH levels (4.5-6.0) adjusted in the medium using 1N HCl/1N NaOH. After incubation for 20 days at 25±2°C, the cell biomass and ephedrine content were analysed as described under sections 5.2.8 and 5.2.10, respectively.

5.2.12 Influence of inoculum concentration

The effect of inoculum concentration on growth was determined with various levels of inoculum (1-7% w/v). Ten day old actively growing cell suspension cultures were used as inoculum. Cells were harvested by centrifugation (Kubota 6700, Japan) at 10,000 rpm at 4°C for 10 minutes under aseptic conditions. The harvested cells were added to preweighed flasks containing 20 ml sterile media, using sterile spatula and the required inoculum concentration was adjusted based on the final weight. Further, 1 ml of cell free supernatant of the actively growing inoculum was also added to each 20 ml shake flask cultures as a conditioner. After 20 days of incubation in an orbital shaker (90 rpm) at 25±2°C under 1000 lux light intensity, the cell biomass was determined as described under section 5.2.8.

5.2.13 Growth curve and production profile

Growth curve studies were carried out in MSSR 4 medium. The inoculum selected after optimisation was added aseptically to the media and incubated at 25 ±2°C in an orbital shaker (Orbitek, India) at 90 rpm, under 1000 lux light intensity (16h photoperiod). Samples were analysed for growth and alkaloid production at regular intervals of 2 days as detailed under sections 5.2.8 and 5.2.10 respectively.

The specific growth rate (μ) and the doubling time (td) were estimated as described earlier under section 4.2.7.

5.2.14 Extraction and isolation of ephedrine

The cells were separated by centrifugation (10,000 rpm for 10 minutes), washed with distilled water and drained fully under vacuum. These cells were used for extraction and isolation of alkaloid.

The extraction of the alkaloid was performed following the method suggested by Yamasaki *et al.* (1973) as detailed below. The cells were moistered with 0.1N HCl and homogenized in a tissue homogenizer (Remi motors, India) and extracted with a mixture of ether: chloroform (3:1). The mixture was made strongly alkaline with potassium carbonate and almost saturated with sodium chloride and kept overnight in an orbital shaker (150 rpm). The extract was then centrifuged (10,000 rpm for 10 minutes) and the residue was repeatedly (3 times) extracted with ether: chloroform mixture. The extracts were pooled and lyophilized in a lyophilizer (Yamato, Japan). The residue after lyophilization was dissolved in small (2 ml) aliquots of chloroform and used for separation by preparative TLC.

The solvent system and the spray reagent for preparative TLC were the same as described under section 3.2.5 previously. About 1 cm width of silica gel corresponds to R_f value 0.56 was scraped off and extracted in methanol. The methanol extract was lyophilized to get pure ephedrine hydrochloride.

5.2.15 NMR spectrum

The ^{1}H NMR spectrum (300 MHz) of the isolated alkaloid from the cells was taken and compared with the spectrum of pure ephedrine (Aldrich Chemicals, USA).

5.3 Results

5.3.1 Presence of latent contamination

In solid culture, no symptoms of contamination was observed even after ten generations of subculturing. The callus was healthy and friable at the time of initiation of suspension culture. On the third day of incubation of suspension culture, turbidity was observed in all the shake flask cultures. On analysis, the rapid increase in turbidity revealed the presence of microbial contamination. The presence of contaminants in all the shake flask cultures and in repeated experiments with fresh callus revealed that it was not accidental contamination, but latent in nature.

5.3.2 Detection and identification of the contaminant

The colonies developed on the plates, for the surface sterilized samples of sterility tests were similar in morphology to those obtained by plating the contaminated suspension culture. All the isolates were picked and identified to their generic level. Interestingly it was observed that all the isolates belonged to *Bacillus* sp (Claus & Berkley, 1986).

5.3.3 Antibiotic sensitivity test

From the results presented in fig.5.1 it is evident that among the ten antibiotics tested, ciprofloxacin and gentamycin showed maximal sensitivity at a concentration of 10 μg each, compared to others on nutrient agar plate (Plate 5).

5.3.4 Minimal inhibition concentration (MIC)

The minimal inhibition concentration of gentamycin and ciprofloxacin were observed as 10 μg ml^{-1} and 12 μg ml^{-1} respectively (Fig.5.2).

5.3.5 Minimal lethal concentration (MLC)

The minimal lethal concentration of gentamycin and ciprofloxacin were observed as 14 μg ml^{-1} and 18 μg ml^{-1} respectively (Fig.5.3).

5.3.6 Control of contaminant bacteria

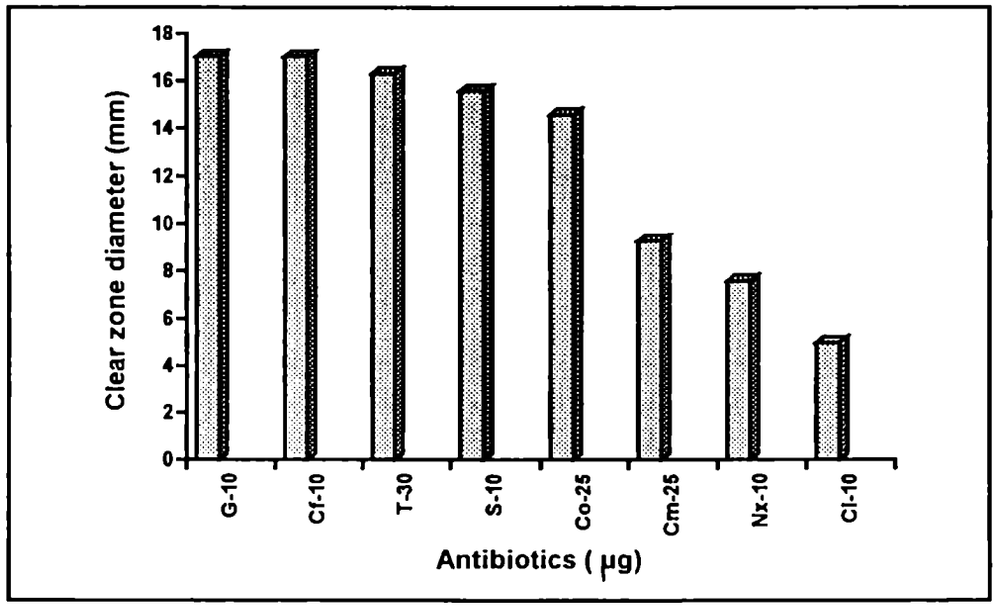
Administration of filter sterilized gentamycin and ciprofloxacin at concentrations of 14 μg ml^{-1} and 18 μg ml^{-1} respectively were proved to be very effective to control the contaminants which occurred as latent contaminant in *S.rhombifolia* and was safe for the plant cells which retained more than 80% viability (Fig.5.4a&b).

5.3.7 Influence of pH

Ephedrine production by *S.rhombifolia* was influenced by the pH of the medium (Fig.5.5). Both the biomass and alkaloid yield were on par over a pH

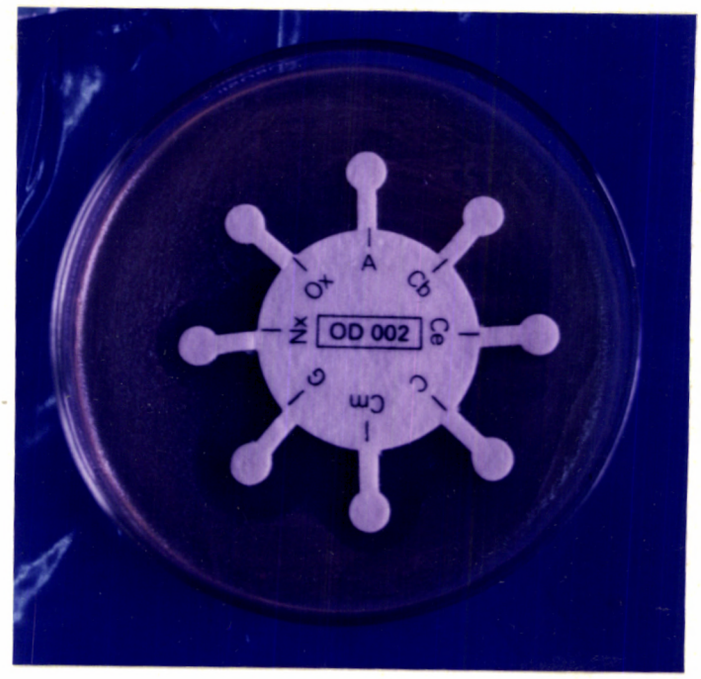
Fig 5.1

Comparative efficacy of sensitive antibiotics against the contaminant *Bacillus* sp.

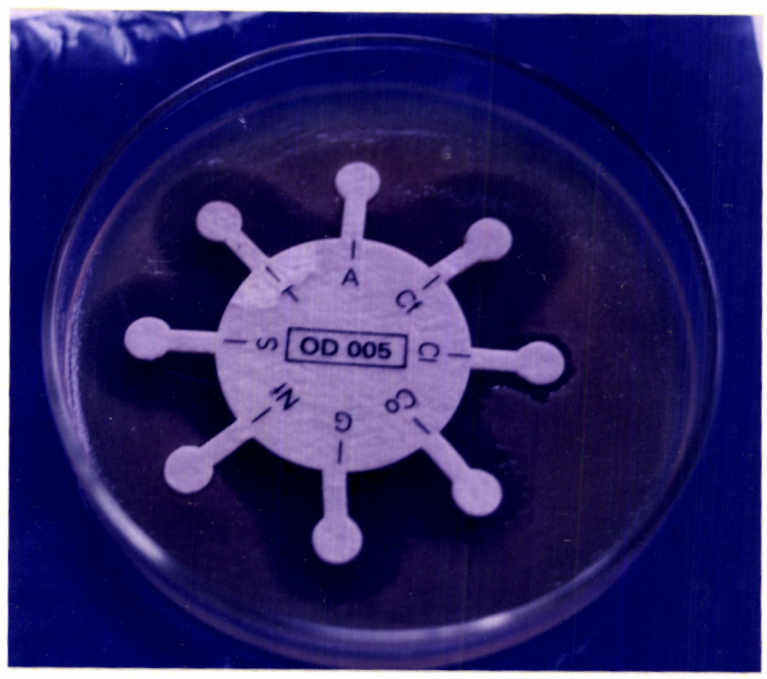


G-gentamycin (10 ug) , Cf-ciprofloxacin (10 ug), T-tetracyclin (30 ug), S-streptomycin (10 ug), Co-co-trimoxazol (25 ug), Cm-co-trimazine (25 ug), Nx-norfloxacin (10 ug), Cl-colistin (10 ug). The data are the mean of 5 replicates. The cultures were incubated at 30^0C for 24 h.

Plate 5 Antibiogram of the contaminant Bacillus sp.



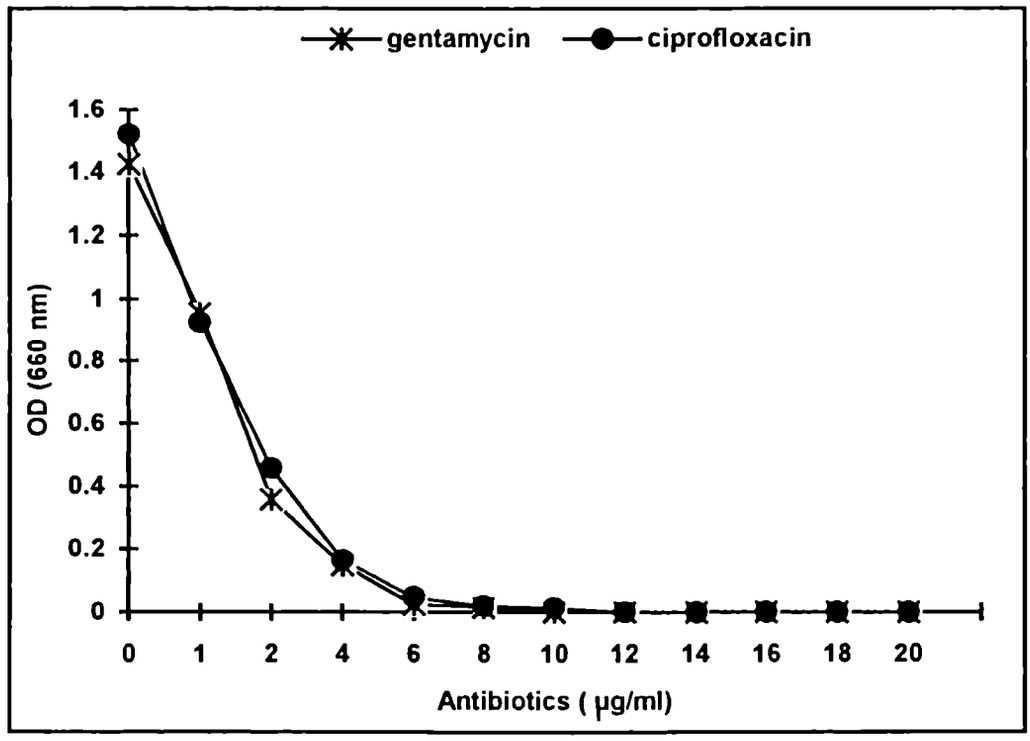
Antibiotic octodisc OD – 002



Antibiotic octodisc OD – 005

Fig 5.2

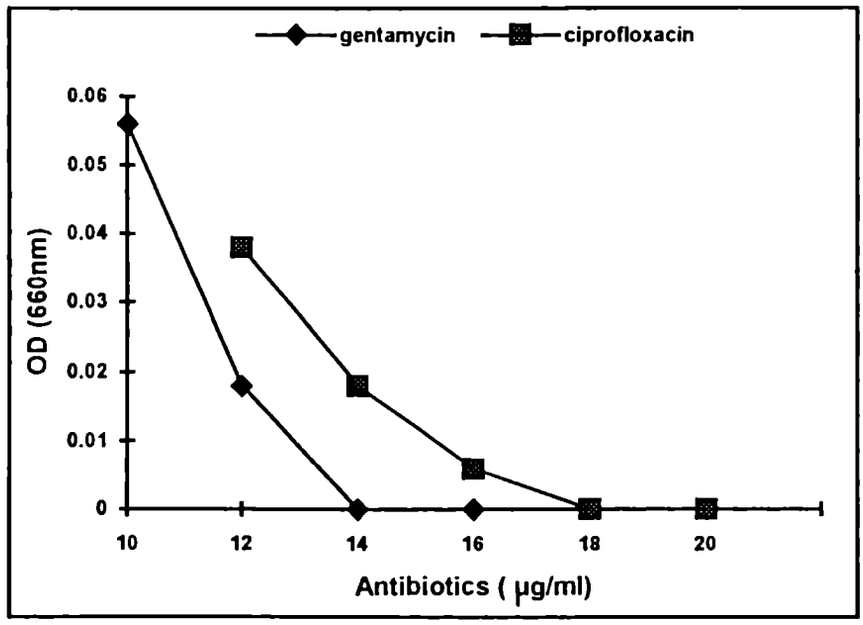
Minimal inhibition concentration (MIC) of gentamycin and ciprofloxacin against the contaminant *Bacillus* sp.



The data are the mean of 5 replicates. The cultures were incubated at 30^{0}C in an orbital shaker (150 rpm) for 24h.

Fig 5.3

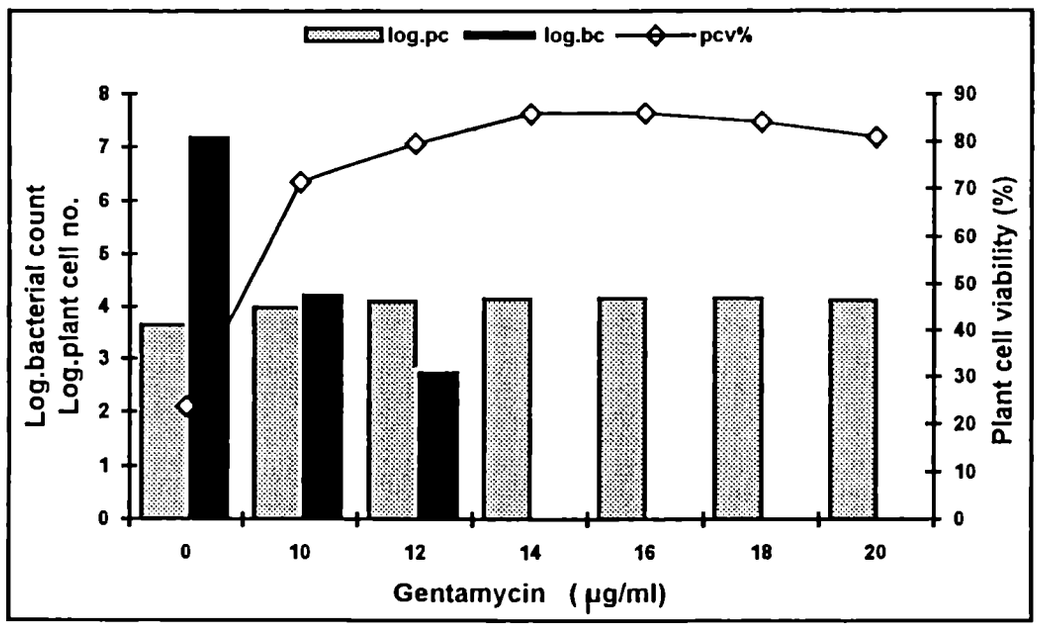
Minimal latent concentration (MLC) of gentamycin and ciprofloxacin against the contaminant *Bacillus* sp.



The data are the mean of 5 replicates. The cultures were incubated at 30°C in an orbital shaker (150 rpm) for 24h.

Fig 5.4 (a)

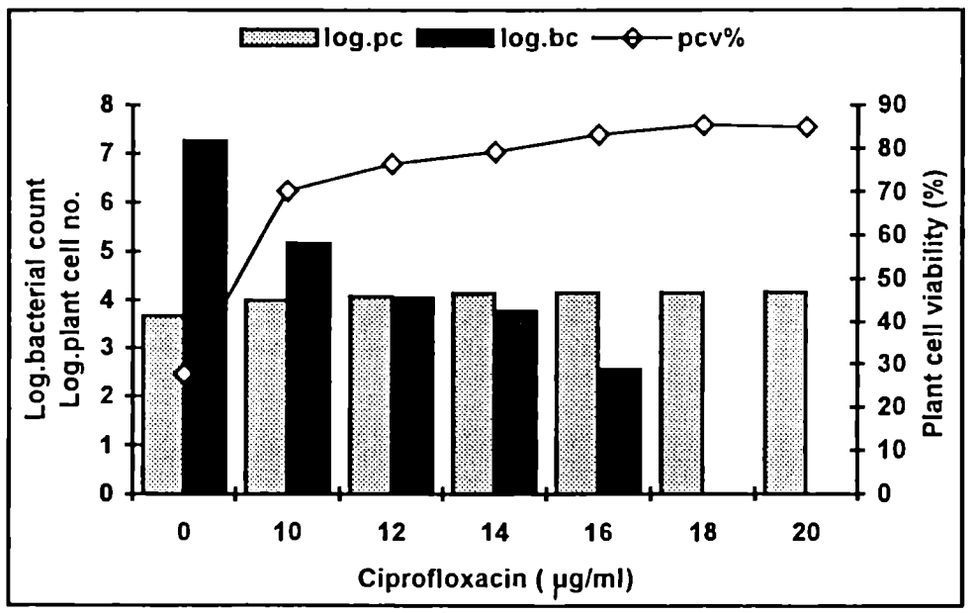
Influence of gentamycin on control of the contaminant *Bacillus* sp in *S.rhombifolia* suspension culture



Data represent average of 5 replicates. Cultures were incubated for 10 days on a rotary shaker (90 rpm) at 25±2^{0}C under 1000 lux light intensity (16h photoperiod).

Fig 5.4 (b)

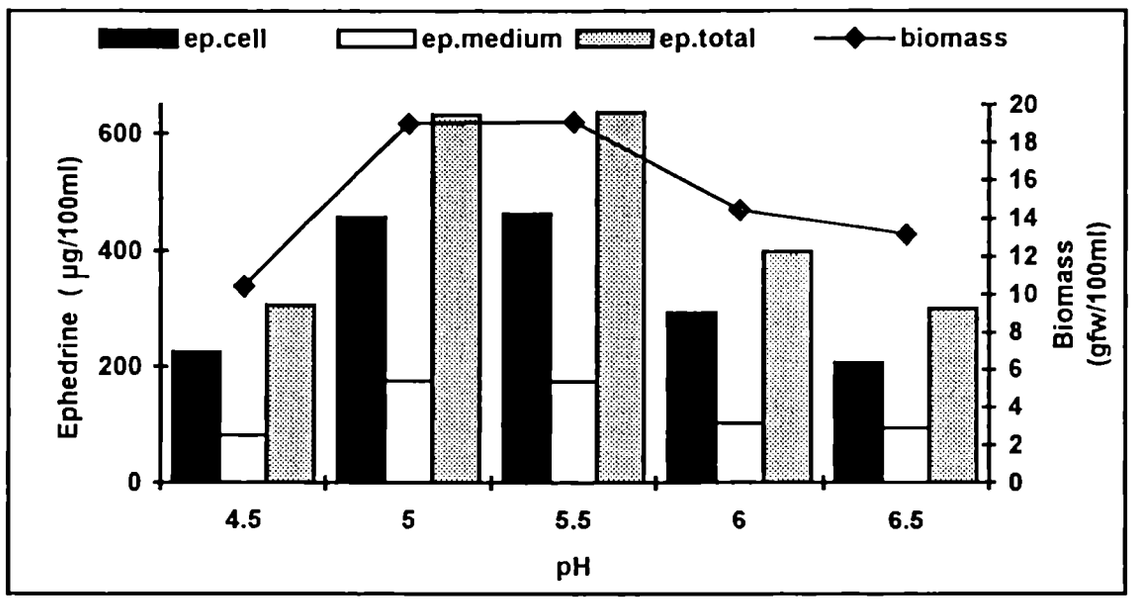
Influence of ciprofloxacin on control of the contaminant *Bacillus* sp in *S.rhombifolia* suspension culture



Data represent average of 5 replicates. Cultures were incubated for 10 days on a rotary shaker (90 rpm) at 25±2^{0}C under 1000 lux light intensity (16h photoperiod).

Fig 5.5

Influence of pH on growth and ephedrine production by *S.rhombifolia* under suspension culture



Data represent average of 5 replicates. The cultures were incubated for 20 days at 25± 2^0C under 1000 lux light intensity on a rotary shaker at 90rpm.

range of 5.0-5.5 although the maximum biomass yield was on pH 5.5 and alkaloid yield on pH 5.0.

5.3.8 Inoculum concentration

From the results presented in fig.5.6 it was inferred that an inoculum concentration of 5% was ideal for optimum growth of culture although inoculum concentration more than 5% showed slight increase in ephedrine yield.

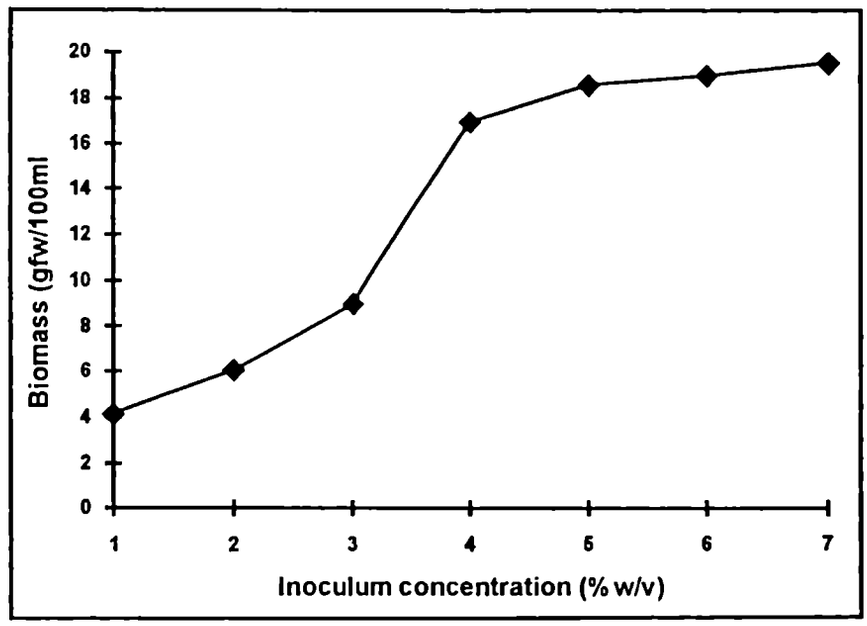
5.3.9 Growth and production profile of *S.rhombifolia* in suspension culture

From the growth profile of *S.rhombifolia* in suspension culture (Fig.5.7) it is evident that the cells grew well in suspension culture (Plates 6 & 7), with a 4 fold increase in cell dry wt. over the growth cycle. The growth curve showed a typical sigmoid pattern with well defined lag (about 5 days), exponential (about 9 days) and stationary phases. The maximum growth was observed on 14^{th} day of culture. The specific growth rate (μ) was estimated to be 0.173 day^{-1} and the doubling time (td) was estimated to be 4 days.

The alkaloid production started at the early logarithmic phase, but alkaloid accumulation was more in the late logarithmic phase (Fig.5.7). The ephedrine production was maximum on the 14^{th} day of culture (649 μg/100 ml culture). Only around 27% of the total alkaloid was released into the medium and the remaining was stored in the cell.

Fig 5.6

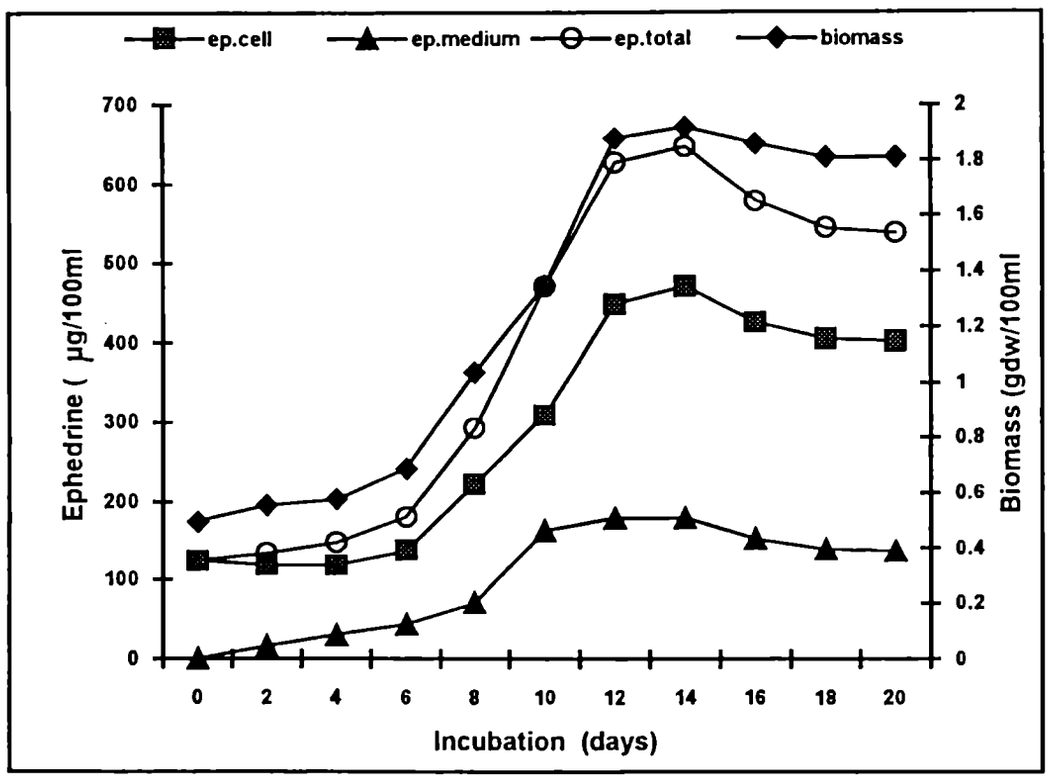
Influence of inoculum concentration on biomass yield of *S.rhombifolia*



Data indicate average of 5 replicates. Cultures were incubated for 20 days at 25±2^{0}C under 1000 lux light intensity on a rotary shaker at 90rpm.

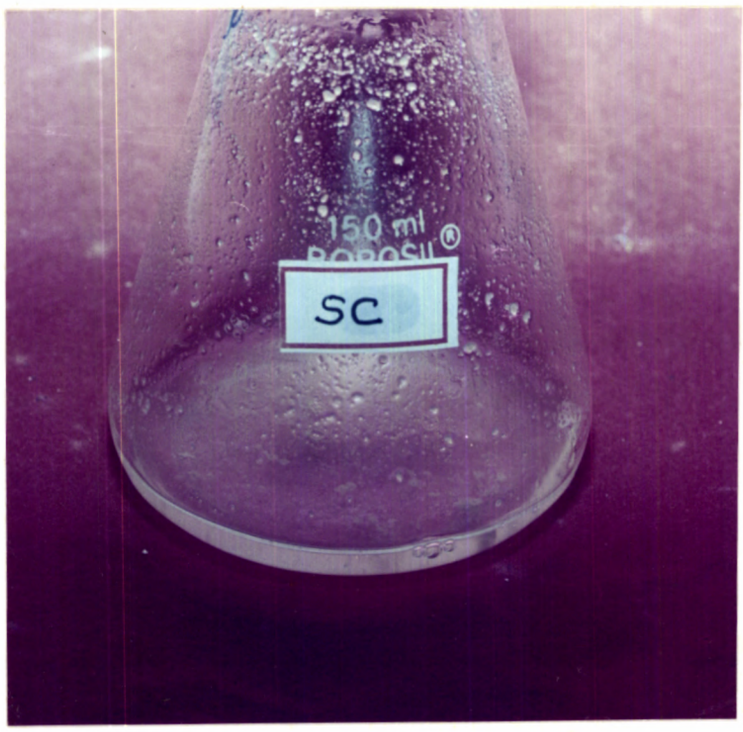
Fig 5.7

Time course experiment on growth and ephedrine productuion by *S.rhombifolia* under suspension culture



Data represent average of 5 replicates. The cultures were incubated at 25± 2^{0}C under 1000 lux light intensity on a rotary shaker at 90rpm. pH 5.5.

Plate 6 Cell suspension culture of *S.rhombifolia*

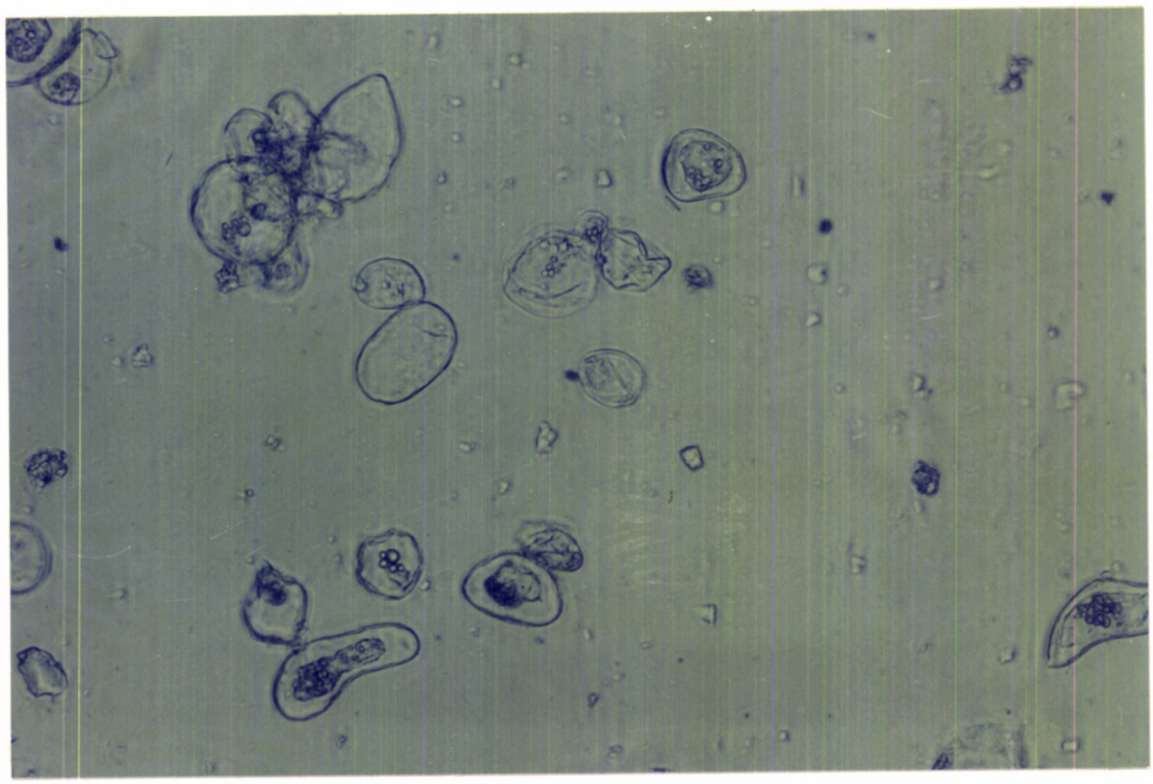


A. At early exponential phase

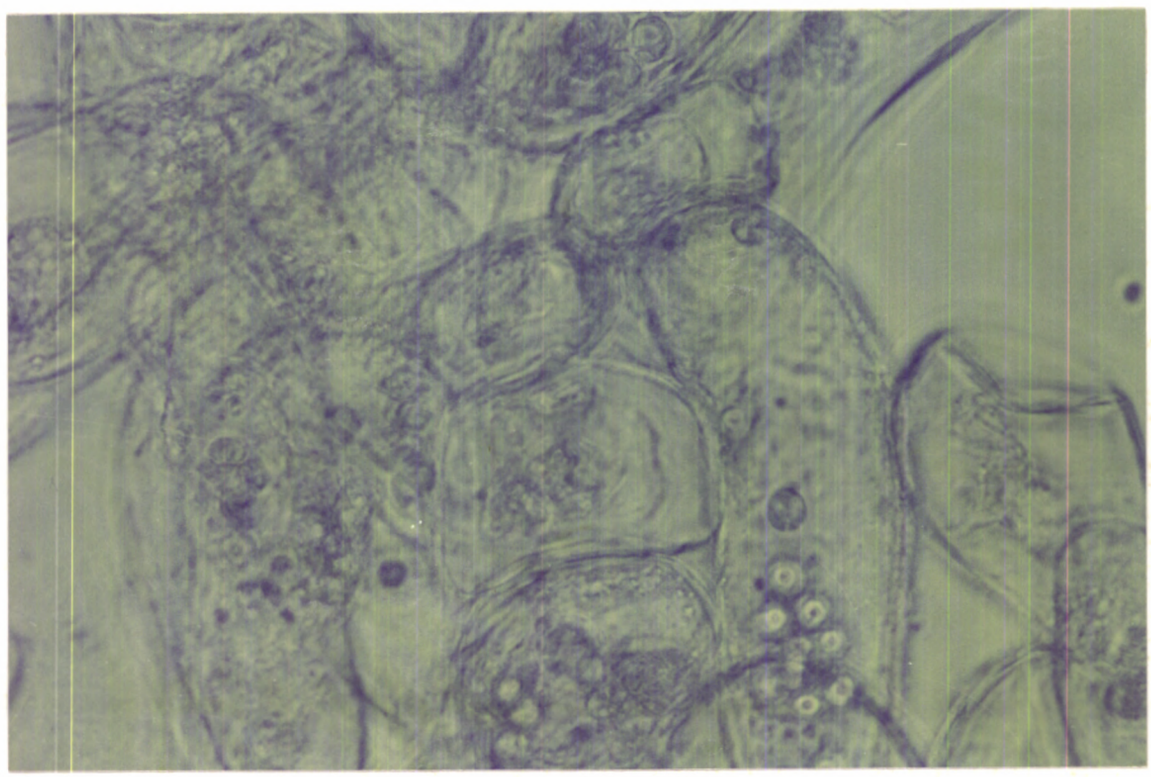
B. At stationary phase

C. Under continuous agitation in orbital shaker

Plate 7 **Cells of *S.rhombifolia* under suspension culture**



A. At early exponential phase (40X)



B. At late exponential phase (100X)

5.3.10 NMR Spectrum

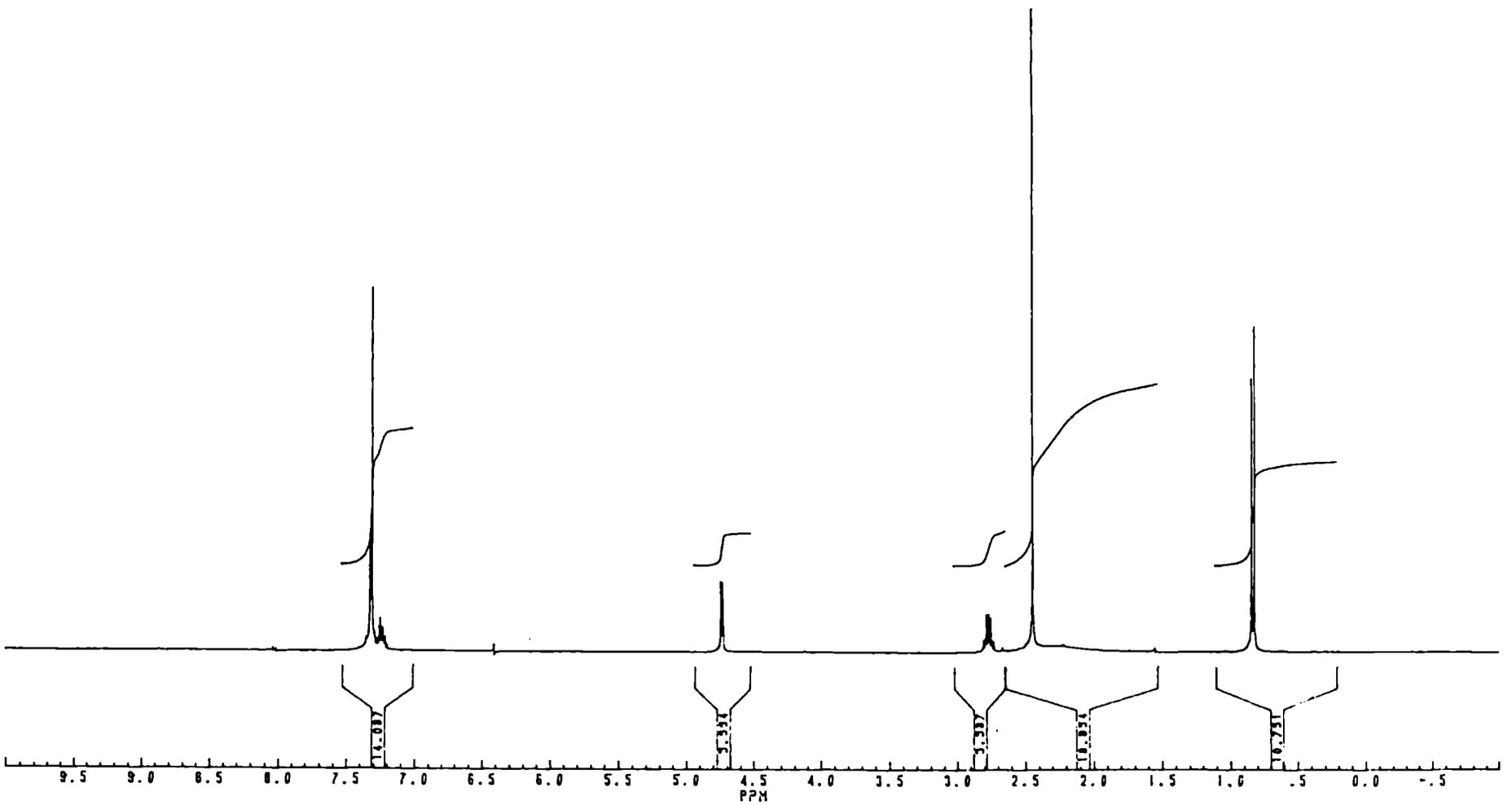
The ^1H NMR spectrum (300 MHz) of the isolated product from the cells was run in $CDCl_3$ which gave signals at δ = 0.9, 2.5, 2.8, 4.8 and 7.3 and the spectral data correlated well with that of ephedrine (Fig.5.8).

5.4 Discussion

Contamination of plant tissue cultures can be subdivided into three types (i) acute contamination at the establishment stage due to incomplete surface sterilization of the explant, (ii) contamination that occurs post establishment, possibly due to an endogenous microflora or poor technique at the subculture stage and (iii) chronic contamination that occurs apparently in batch of cultures after an extended period of supposedly axenic growth (Constantine, 1986). There are conflicting views in the literature as to the seriousness of contamination that occurs post establishment. Constantine (1986) expressed the opinion that latent contamination is the biggest single problem faced by all micropropagation laboratories.

The sudden appearance of visible growth of bacterial contamination at later *in vitro* stages (after many subcultures) has been attributed to contaminants which had been introduced with the initial plant material. In the present investigation, in sterility test using the sap from the surface sterilized explants in nutrient agar medium indicated the presence of latent bacterial contamination in the original plant material used. The apparent lag period between the introduction and apparent visible growth of contaminants has been explained by the presence

Fig. 5.8 NMR spectrum of the isolated ephedrine from *S - rhombifolia* cells



of latent bacteria, which need either to adapt to the *in vitro* environment or require a change in the *in vitro* environment for growth (for example transfer to another media and/or a change in growth/room temperature) (Cornu & Michel 1987; Fisse *et al.* 1987). In the present study, the sudden change in the *in vitro* environment while transfering friable callus from static culture to liquid culture might be the reason for the expression of latent bacteria in suspension culture. Moreover, in liquid culture, every cell/small aggregates of cell was in direct contact with nutrient medium which also might have favoured the growth and expression of latent bacteria.

Many investigators have described the antibiotic sensitivity of bacteria isolated from plant tissue cultures (Cornu & Michel, 1987; Leggatt *et al.* 1988) or included antibiotics in the plant growth medium to suppress or eliminate bacterial contaminants (Philips *et al.* 1981; Mathias *et al.* 1987; Pod Wyzynska & Hempel 1987). Further, most of them have observed bactericidal or bacteriostatic effect of their antibiotic treatment on contaminants but were subsequently criticized for not assessing plant cultures for long enough to make certain of the success of their treatment (Debergh & Vanderscharge, 1988). Whereas, some have reported that certain antibiotic treatments had no effect on contamination (Renstle *et al.* 1988; Leifert *et al.* 1991). In addition, many antibiotics have been found to be phytotoxic to plants *in vivo/in vitro* and can therefore only be incorporated into plant growth media for limited periods of time (Cornu & Michel, 1987; Mathews 1988; Falkiner 1990, Leifert *et al.* 1991).

Several workers have reported the successful administration of comparatively high concentration of antibiotics in the medium to control the contaminants. Administration of gentamycin into plant growth media at a concentration of 200 mg l^{-1} in combination with rifampicin (100 mg l^{-1}) and carbenicillin (200 mg l^{-1}) for 72 days were found to be successful and not phytotoxic and were free of contaminants even after two years of treatments (Philips *et al.* 1981).

In the present investigation, pretreatment of the callus tissue with the sensitive antibiotics for 30 minutes resulted in the inhibition of the growth of the bacteria but did not eliminate them. Hence in the later generations of subculturing, the contaminant bacteria again appeared whereas incorporation of antibiotics (14 µg m l^{-1} gentamycin and 18 µg ml^{-1} ciprofloxacin) into the medium, could attribute to the effective control of the contaminant. A very low concentration of 14 µg m l^{-1} gentamycin and 18 µg ml^{-1} ciprofloxacin were adequate for the effective control of the contaminant as against the high dozes of antibiotics reported earlier.

In many cases, the tissue culturists use the antibiotics without screening for antibiotic sensitivity and without determining the MIC and MLC of the antibiotics, which might be the reasons for the low success, need for the use of high dosage and consequent phytotoxicity reported earlier in many instances. The antibiotics are expensive and moreover no one compound is effective against all possible types of contaminating organisms. Further, it is a widely accepted fact

that antibiotics are preferred when explanted material contains concealed microorganisms that are a major obstacle to *in vitro* culture and difficult to eliminate by alternative means.

Gentamycin is highly specific in inhibiting the protein synthesis by binding to 30S subunit of the bacterial ribosome and cause misreading of the mRNA. The action of ciprofloxacin is complex but includes inhibition of DNA gyrase, the enzymes that maintains helical twists in DNA and thereby attribute to bactericidal activity. The greater control of bacterial infection by antibiotics in liquid medium may be due to increased surface contact and increased uptake of antibiotics into internal tissues (Leifert *et al.* 1991). Further, the alternative use of the two antibiotics were recommended in order to prevent the development of resistance against a particular antibiotic. Under suspension culture, a lower pH of 5.0-5.5 was ideal for maximum growth and alkaloid production of *S.rhombifolia* compared to solid culture. The pH of a culture varies according to the plant species and stage of culture. In *Helianthus annus* suspension culture, the pH of the culture was raised from 5.8 in the lag phase to 6.8 in the stationary growth phase (Endress, 1994) whereas, in *Morinda citrifolia* cultures, the pH of the culture was decreased to 4.0 in the log phase from a pH of 5.4 in the lag phase and again raised to 5.1 in the stationary phase (Endress, 1994). As the pH change is not uniform in all the cultures, the optimization of ideal pH for maximum growth and production is highly essential for each and every system.

Unlike microbial cultures, the plant cell cultures require a higher inoculum concentration for satisfactory growth. The minimal amount needed is 2-3 g/100 ml medium (Endress, 1994). The growth of plant cultures depends on a minimum cell density and with *Acer* sp. it was reported to be 9-15x10^3 cells ml^{-1} (Street, 1977). A very high inoculum concentration of 10% (w/v) was also reported in some systems (Johnson, 1993). In the present investigation, 5% inoculum level (w/v) using 10 day old actively growing culture alongwith addition of 1 ml of cell free supernatant yielded maximum growth. With further increase, the growth remained almost static. The addition of 1 ml cell free supernatant might have a conditioning effect, as reported earlier (Ludwig *et al.* 1985; Teasdale & Richards, 1991).

A time course study indicated that the cells followed a sigmoid growth pattern similar to that of a microbial system with a lag phase of 5 days, a logarithmic phase of 9 days followed by a stationary growth phase. Eventhough the alkaloid production was started at the early logarithmic phase, alkaloid accumulation was more in late logarithmic phase and the content in the medium also increased correspondingly. There was no further increase in the alkaloid content during the stationary phase of cell growth. Only 27% of the total alkaloid was excreted into the medium.

From the growth profile, it is evident that *S.rhombifolia* suspension culture is a slow growing system with an estimated specific growth rate (μ) of 0.173 day^{-1}. This suggests that the biomass increased by about 17.3% in one day during

the exponential growth phase. But in fast growing experimental systems such as *N.tabacum* and *Daucus carota*, a very high specific growth rate (μ) of 1.1 day^{-1} (Noguchi *et al.* 1982) and 0.22 day^{-1} (Madusudhan *et al.* 1995) respectively were reported. The doubling time (td) of cells in *S.rhombifolia* suspension culture was estimated to be 4 days.

In the spectral data of the isolated product, the signal at 0.9 (3H, d) which appeared as a doublet can be attributed to $-CH-CH_3$ and $-NH-CH_3$ appeared at δ2.5 (3H,S). The signal at δ2.8 (1H,m) was due to $CH-CH_3$ proton and the benzylic Ar-CH-OH appeared at 4.2 (1H,d) as doublet. As expected, the aromatic H's appeared at δ7.3 (5H,m). These observations confirm that the isolated compound is ephedrine.

CHAPTER VI

Studies on yield enhancement

Eventhough plant cell culture technology offers a great potential for the production of secondary metabolites, the major problem associated with plant cell cultures is that the cultures initially show a productivity which is far below that needed to make any exploitation economically feasible (Fowler 1983 & 1986; Yeoman *et al.* 1990). Much effort has been put into the development of methods to enhance the productivity of plant cells. A number of parameters have been identified which potentially influence the productivity in plant cell cultures. These include

i) Selection of high yielding cell lines by using specific mutagens

ii) Inducing nutrient limitation

iii) Addition of specific elicitor compounds

iv) Use of permeation agents

v) Supply of precursors

6.1 Selection of high yielding cell lines using specific mutagens

6.1.1 Introduction

Plant tissue and cell cultures are excellent systems for the isolation of mutants over the use of a whole plant. Both physical and chemical mutagens can change the DNA and therefore induce mutations.

Mutant cell lines are useful for basic plant genetic research and for enhanced production of high value phytochemicals (pharmaceuticals, food additives, colours

etc.) Cells in suspension cultures are physiologically homogeneous and cells occur in small clumps. Suspension cultures are more advantageous over callus cultures for mutant selection. Maximum number of cells can be challenged by the mutagenic agent and several million cells can be screened easily, within a small volume of culture medium.

In the present investigation, the cells were treated with both chemical and physical mutagens to select the high yielding cell lines, if any. The strategy adopted was repeated mutagenic treatment, facilitating greater chances of recovery of desirable mutations.

6.1.2 Materials and methods

Mutagenic studies were carried out on *S.rhombifolia* using the chemical mutagens EMS (Ethyl Methane Sulphonate) and AO (Acridine Orange) and the physical mutagen, ultraviolet radiation (UV).

6.1.2.1 Mutagenesis of the explants using chemical mutagens (EMS&AO)

Surface sterilized explants were treated with filter sterilized EMS (v/v) and A.O (w/v) at different concentrations (0.2%, 0.4%, 0.6%, 0.8%, 1.0% and 2.0%). In order to get uniform penetration of the chemicals, the flasks containing explants in the above solutions were incubated, on a gyratory shaker, for various incubation periods of 30, 60 and 90 minutes. After treatment, the explants were washed repeatedly with sterile distilled water to make free of mutagenic agents, and cultured in the MSSR-4

medium for 30 days at 25±2°C under 1000 lux illumination. The fast growing callus lines were selected and subjected to repeated mutagenic treatment.

6.1.2.2 Mutagenesis of the cell suspensions using chemical mutagens (EMS & AO)

i) The following media were prepared

Medium A - MSSR-4 + 3% sucrose

Medium B - Medium A + 0.02% EMS (v/v)/AO (w/v)

Medium C - Medium A + 1.2% agar

Medium D - Medium A + 1.0% agar.

ii) Medium A was inoculated with 1 g each of fast growing callus lines selected after treatment with EMS/AO as described under section 6.1.2.1.

iii) The cultures were grown on a rotary shaker (90 rpm) at 25±2°C under continuous light (1000 lux) for a period of ten days/mid log phase.

iv) The cells were subcultured at regular intervals on the same medium.

v) Filtered the cells through nylon cloth to remove large clumps.

vi) The cells in mid log phase were transferred to medium B, containing mutagens (EMS 0.02% v/v and AO 0.02% w/v) and maintained in medium B for 6h on a gyratory shaker (90 rpm) at 25±2°C.

vii) After incubation, the cells were washed with fresh culture medium, 3-4 times, to remove EMS/AO completely and resuspended the cells in fresh medium A.

viii) Cells were centrifuged at 5000 rpm for 20 min. and resuspended in minimal volume of medium A.

ix) Determined the viability of cells by dye exclusion technique using Evan's blue.

x) The packed cells were diluted 1:1 with medium C at 40-45°C, which resulted in 0.6% agar concentration.

xi) 1-2 ml of the suspension was plated on prepared plates containing medium D. Allowed the plates to stand for 30 min.

xii) Sealed the plates with parafilm and incubated for 45 days at 25±2°C under 1000 lux light intensity.

xiii) Fast growing colonies were picked and subcultured in test tubes containing solid MSSR-4 medium for 30 days at 25±2°C under 1000 lux light intensity.

xiv) Ephedrine content in the suspension culture of the selected cell lines were analysed as detailed under section 5.2.10 and further selection was made based on ephedrine yield.

xv) The selected cell lines were subjected to a higher doze of 0.04% mutagen (EMS & AO) treatment as performed earlier and further selections were made for maximum ephedrine production.

6.1.2.3 Mutagenesis using ultraviolet irradiation

The high yielding cell lines selected from chemical mutagen treatments were further subjected to mutagenesis using ultraviolet irradiation.

i) The following media solutions were prepared

Medium A - MSSR-4 + 3% sucrose

Medium B - Medium A + 1.2% agar

Medium C - Medium A + 1.0% agar.

ii) Prepared media were inoculated with 1 g each of the high yielding mutant cell lines selected after chemical mutagen treatment on medium A.

iii) The cultures were grown on a gyratory shaker (90 rpm) at 25±2°C under continuous light (l000 lux) for a period of ten days/mid log phase.

iv) The cells were subcultured at regular intervals on the same medium.

v) Filtered the cells through the sieve/nylon cloth to remove large clumps.

vi) Resuspended the cells in minimal volume of medium A.

vii) The packed cell suspension was taken in a sterile petridish with the lid open and exposed for 60 min. to UV irradiation from a 20W UV lamp (Philips, Holland) kept at a distance of about 15 cm. Care was taken to switch on the UV lamp at least 30 min. before the commencement of the experiment in order to stabilize the emission.

viii) After irradiation, the packed cell suspension was diluted with medium B (1:1), at 40-45°C, which finally resulted in 0.6% agar concentration.

ix) 1-2 ml of the suspension was plated on prepared plates containing medium C. Allowed the plates to stand for 30 min.

x) Sealed the plates with parafilm and the fast growing colonies were subcultured in test tubes containing solid MSSR-4 medium and incubated for 30 days at 25±2°C under 1000 lux light intensity.

xi) Ephedrine analysis of the selected lines were made in suspension culture as detailed under section 5.2.10 and selection was made based on production.

6.1.2.4 Ephedrine production by the mutant cell lines

The cell lines, selected after repeated mutagenesis with EMS, AO & UV irradiation, were evaluated for alkaloid production using MSSR-4 medium at pH 5.5. The inoculation and incubation of the culture and the alkaloid production capacity were determined as mentioned under sections 5.2.3 and 5.2.10, respectively.

6.1.3 Results

6.1.3.1 Mutagenesis of the explants using chemical mutagens

The explants treated with EMS recorded callus initiation up to 1% concentration compared to AO which showed only up to 0.8%. Treatment of explants with mutagens, for 60 min. was observed to be ideal as the treatment caused more lethality. Though, lethality was more for 90 min. treatment, survival percentage was zero for AO and 10% for EMS. Further, the calli initiated with concentrations up to 0.6% only survived in the case of both EMS & AO (Table 6.1.1). Eight fast growing calli were selected from the chemical mutagen treatment.

Table 6.1.1

Induction of callus by explants of *Sida rhombifolia* after treatment with chemical mutagens

Mutagen	Concen-tration	Callus initiation (%)			Survival (%)		
	(% v/v)	30 min	60 min	90 min	30 min	60 min	90 min
EMS	0.2	70	60	50	40	30	10
	0.4	60	50	30	20	20	0
	0.6	40	40	20	10	0	0
	0.8	20	10	0	0	0	0
	1.0	10	0	0	0	0	0
	2.0	0	0	0	0	0	0
	(% w/v)	30 min	60 min	90 min	30 min	60 min	90 min
AO	0.2	80	60	40	50	30	0
	0.4	60	40	20	30	10	0
	0.6	40	30	10	20	0	0
	0.8	10	20	0	0	0	0
	1.0	0	0	0	0	0	0
	2.0	0	0	0	0	0	0

EMS – Ethyl Methane Sulphonate, AO – Acridine Orange. The percentage was worked out of 10 replicates. The mutagen treated leaf explants were inoculated on MSSR-4 medium and incubated at 25±2°C at 1000 lux light intensity.

6.1.3.2 Mutagenesis of the cell suspension using chemical mutagens (EMS & AO)

Four cell lines from 0.02% EMS mutagenesis and 3 cell lines from 0.02% AO mutagenesis were selected based on their fast growth.

The seven selected cell lines were subjected to further treatments with higher concentration (0.04%) of EMS & AO and 5 cell lines were finally selected based on their rapid growth.

6.1.3.3 Alkaloid production by the mutant cell lines

Data presented in Table 6.1.2 evince that the cell line SRM 1.1.1 had the maximum biomass (18.7 g/100 ml) and ephedrine yield (1.1 mg/100 ml), with a 27% increase compared to that of the untreated control. Among others, the cell line SRM 1.1.2 yielded about 7% increase in ephedrine production while all the other selected mutant lines (SRM 2.1.1, SRM 7.1.1 & SRM 7.2.1) showed reduced yield of ephedrine. However, the cell line SRM 2.1.1 excreted about 47% (0.3 mg/100 ml culture) of its total alkaloid into the medium as against 23% (0.2 mg/100 ml culture) in the control, eventhough the total production was about 19% less compared to the control.

6.1.3.4 UV mutagenesis

The high yielding cell lines selected after chemical mutagenesis, SRM 1.1.1 and SRM 1.1.2 (Plate 8), were further subjected to UV mutagenesis and 4 cell lines were selected and evaluated for ephedrine production. From among the mutants

Table 6.1.2

Biomass and alkaloid yield of the selected mutant lines *S.rhombifolia* after repeated chemical mutagenesis

Mutant lines	Biomass gfw/100 ml culture	Ephedrine (mg/100 ml culture)		
		Cell	Medium	Total
SRM 1.1.1	18.65	0.84	0.24	1.08
SRM 1.1.2	17.00	0.69	0.22	0.91
SRM 2.1.1	14.60	0.37	0.33	0.69
SRM 7.1.1	16.05	0.59	0.19	0.79
SRM 7.2.1	14.15	0.47	0.20	0.67
Control (not treated with chemical mutagen)	18.10	0.65	0.20	0.85

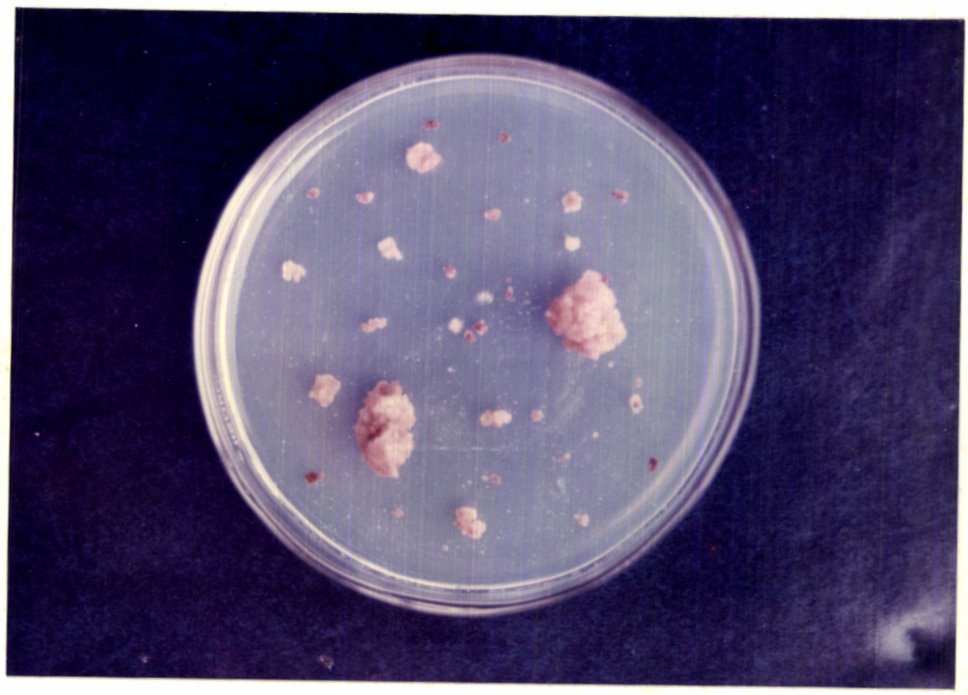


Plate 8 Plated mutant cell lines of *S.rhombifolia*

tested, one mutant line viz., SRM 1.1.1.1 yielded around 32% more alkaloid compared to the untreated control (Table 6.1.3). All the others were relatively poor yielders of ephedrine.

6.1.4 Discussion

The cultured plant cells provide excellent material for mutagenic studies since the use of single cells obtained from liquid suspension cultures or of isolated protoplasts allow quantitative radiation dosimetry and analysis of single cell survival. The advantages of using toxic agents for selection is that majority of the wild type cells are killed, while the desired variant survives the treatment.

In the present study, five mutant cell lines were selected after repeated mutagenesis with chemical mutagens, Ethyl Methane Sulphonate and Acridine Orange. Out of these, two lines viz. SRM 1.1.1 & SRM-1.1.2 showed enhanced ephedrine yield compared to the unirradiated control. Interestingly, one line viz. SRM-2.1.1 released about 47% of the total alkaloid produced, into the medium.

Quesnel and Ellis (1989) reported selection of UV-tolerant cell line of *Nicotiana* and *Anchusa* which exhibited higher levels of phenolics. The mutant line SRM-1.1.1 when subjected to UV-irradiation, a mutant line viz. SRM-1.1.1.1 which exhibited 32% more ephedrine production capacity than the unirradiated check could be selected.

Table 6.1.3

Growth and alkaloid production of the selected mutant cell lines by UV mutagenesis.

Mutant lines	Biomass gfw/100 ml culture	Ephedrine (mg/100 ml culture)		
		Cell	Medium	Total
SRM 1.1.1.1	17.2	0.84	0.28	1.12
SRM 1.1.1.2	14.8	0.57	0.22	0.79
SRM 1.1.2.1	15.6	0.43	0.27	0.69
SRM 1.1.2.2	14.0	0.46	0.24	0.69
Control (unirradiated)	18.1	0.63	0.22	0.85

Based on the results obtained in this study, it is concluded that repeated mutagenesis, involving both chemical and physical agents can be successfully employed to evolve not only high yielding mutant lines but also mutants with desirable attributes such as increased permeability for release of its secondary metabolites into the medium, which is a serious constraint in adopting plant cell culture for secondary metabolite production.

6.2 Yield enhancement studies by inducing nutrient stress

6.2.1 Introduction

Cultured plant cells are usually supplied with all essential minerals, vitamins and carbon source for vigorous growth and active primary metabolism. However, the secondary metabolites are almost invariably produced most vigorously by senescent or slow growing cells (Dziezak, 1986). Hence the general approach is to slow down the growth by limiting the supply of sugar, phosphorus or nitrogen (Johnson, 1993). The cultural conditions which promote high rate of cell division are commonly not conducive to a maximum rate of secondary product formation (Rokem & Goldberg, 1985). Low phosphate levels stimulated cinnamoyl putrescine accumulation in *Nicotiana tabacum* cell suspension by 3-4 fold (Schiel *et al.* 1984). The effects of other nutrients seem less predictable, but substantial changes in product levels can also be produced (Johnson, 1993).

In this study, effect of phosphate, nitrate and sucrose stress in the medium on ephedrine production by the cell cultures of *S.rhombifolia* Linn. ssp. *retusa* (Linn.) Borssum. was evaluated.

6.2.2 Materials and methods

6.2.2.1 Nutrient stress on ephedrine production in *S.rhombifolia* cell cultures

The strategy adopted for inducing nutrient stress was that the actively growing cells were transferred on tenth day from the growth medium and the ephedrine production was assessed on 14^{th} and 18^{th} day as detailed under section 5.2.10.

6.2.2.2 Phosphate stress

Impact of phosphate stress on ephedrine production was studied under two conditions – one without H_2PO_4, and the second one with 50% H_2PO_4 (0.65 m.eq. compared to 1.3 m.eq in the control medium).

6.2.2.3 Nitrate stress

Nitrate stress on ephedrine yield was evaluated under two conditions – one without nitrate and the 2^{nd} one with 50% NO_3 (19.7 m.eq. as against 39.4 m.eq. in the control medium.

6.2.2.4 Sucrose stress

Influence of sucrose stress, on ephedrine yield was studied under two stress conditions, the one without sucrose and the second one with 50% sucrose (1.5% against 3.0% in the control medium).

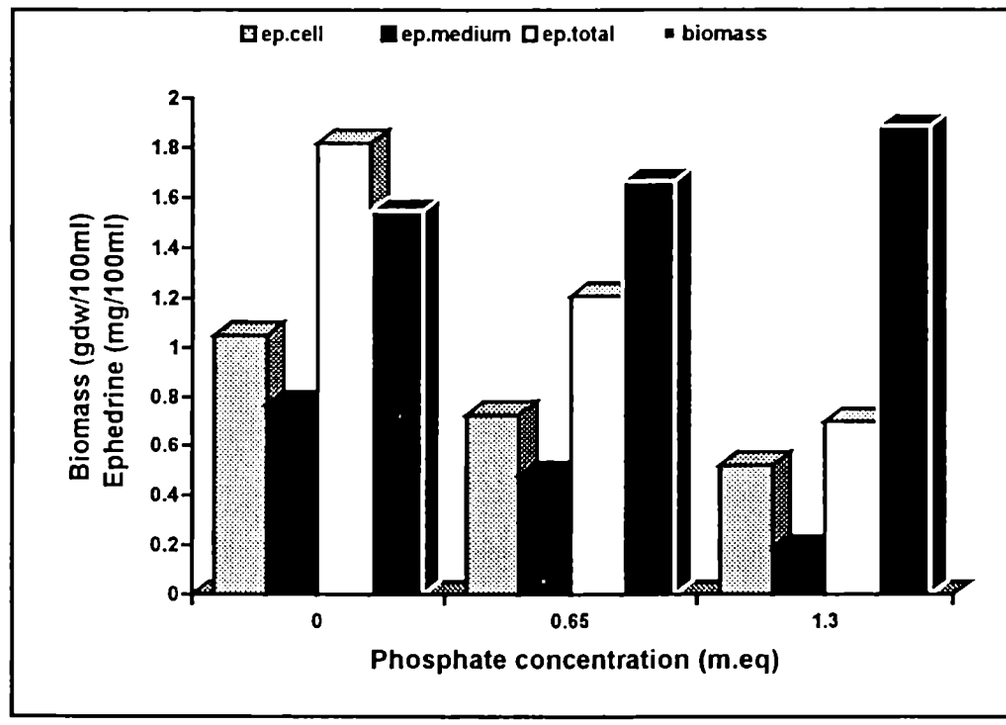
6.2.3 Results

6.2.3.1 Influence of phosphate stress on ephedrine production

Elimination of phosphate in the medium resulted in a gradual increase in ephedrial production (Fig.6.2.1) After 14 days of culture, the ephedrine content was 1.8 mg/100 ml culture, 2.6 times higher than the control while reduction in initial

Fig 6.2.1

Influence of phosphate stress on biomass and ephedrine yield of *S.rhombifolia* cell culture



Data are the average of 5 replicates. Cultures were incubated at 25±2^{0}C under 1000 lux light intensity on a rotary shaker at 90rpm. Actively growing cells in the growth medium were transfered on 10th day of culture to the stress medium and production was assessed after 4 days of further incubation.

phosphate level resulted in 1.73 fold increase in alkaloid yield. There was no further increase in ephedrine production on 18th day, but a slight reduction was noticed. Further, the % release of the alkaloid into the medium was also observed to be high (42% as against 25% in the control) under stress conditions.

6.2.3.2 Influence of nitrate stress on ephedrine production

Complete elimination of nitrate in the medium enhanced 31% increase in total ephedrine (Fig.6.2.2). Though the influence was positive, the yield enhancement was less compared to phosphate stress induction. The improvement in the percent release of the compound into the medium was only marginal (by 7%) whereas, half the conc. of nitrate effected 46% increase in total ephedrine.

6.2.3.3 Influence of sucrose stress on ephedrine production

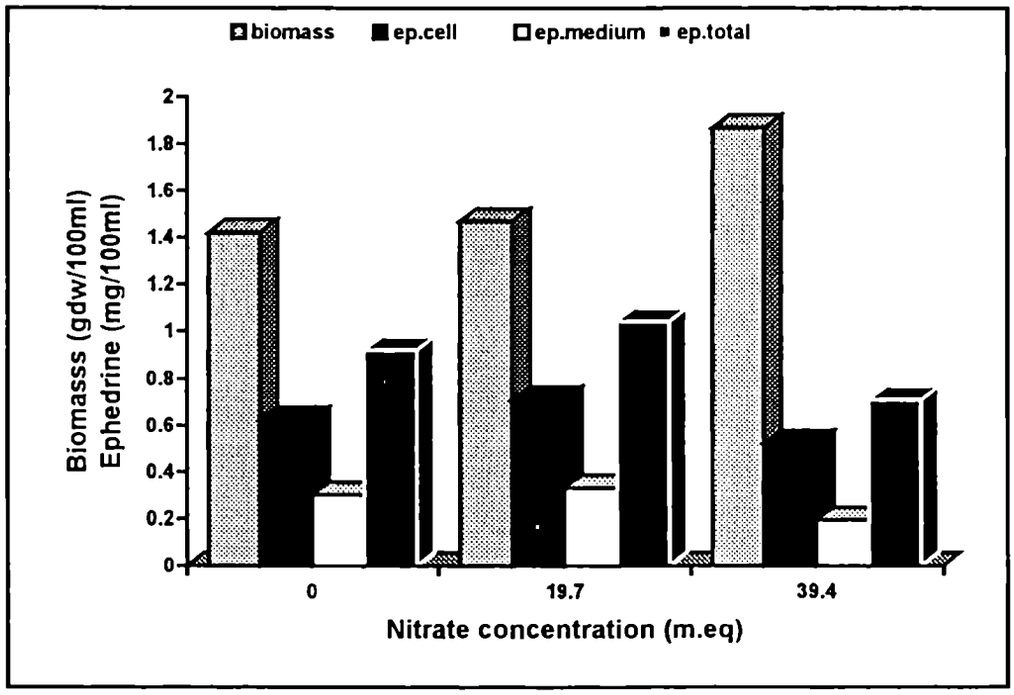
When sucrose was deleted from the medium, about 29% increase in total ephedrine was observed on 14th day of culture, whereas when 1.5% was administered in the medium, only about 14% increase in total ephedrine yield was recorded (Fig.6.2.3). The response was more on 14th day rather than on 18th day.

6.2.4 Discussion

Plant cell cultures are usually grown heterotrophically using a simple sugar as carbon source, an inorganic supply of other nutrients and vitamins, for vigorous growth and active primary metabolism. It has been observed that the secondary products tend to accumulate at the end of growth phase (Johnson, 1993). Therefore,

Fig 6.2.2

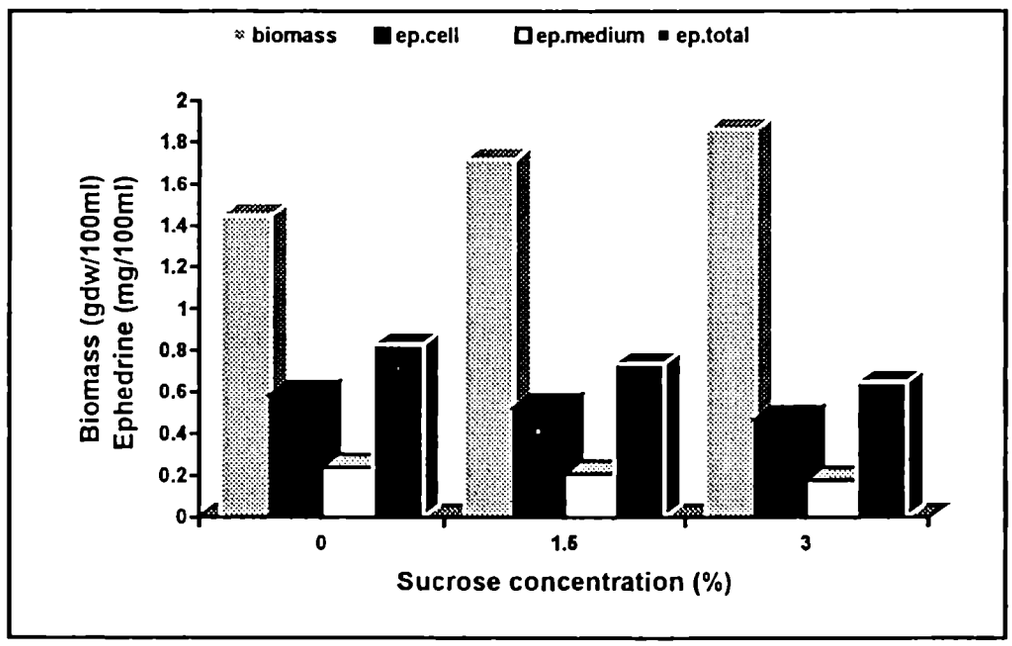
Influence of nitrate stress on biomass and ephedrine yield of *S.rhombifolia* cell culture



Data indicate average of 5 replicates. Cultures were incubated at 25±2°C under 1000 lux light intensity on a rotary shaker at 90rpm. Actively growing cells in the growth medium were transfered on 10th day of culture to the stress medium and production was assessed after 4 days of further incubation.

Fig 6.2.3

Influence of sucrose stress on biomass and ephedrine yield of *S.rhombifolia* cell culture



Data indicate average of 5 replicates. Cultures were incubated at 25±2°C under 1000 lux light intensity on a rotary shaker at 90rpm. Actively growing cells in the growth medium were transfered on 10th day of culture to the stress medium and production was assessed after 4 days of further incubation.

the general approach has been to slow down the growth by limiting the supply of sugar, nitrogen and phosphorus.

The carbon source, for example, sucrose has been shown to influence the production of phytochemicals. Alkaloid level in cultured cells of *Catharanthus roseus* fluctuated in response to varied levels of sucrose in the growth medium (Zenk *et al.* 1977). Lowered sucrose concentration stimulated coumarin synthesis in *Nicotiana tabaccum* (Okazaki *et al.* 1982). In the present study, there was only 29.4% increase in ephedrine yield in sucrose depleted medium whereas ncrease in sucrose concentration, above 3%, enhanced the biosynthesis of phytochemicals in plant cell cultures (Dicosmo & Towers, 1984), which might be due to osmotic stress. The positive effect observedunder sucrose stress might be due to nutrient stress.

The media for plant cell culture usually provide nitrogen as a mixture of NO_3 and NH_4^+salts. When these sources are altered, either alone or in combination or when their ratio is altered or when the levels are changed, they influence the growth and product formation (Jessup & Fowler, 1976; Dougall, 1980).

In most of the cases, a decrease/depletion of nitrogen led to an enhanced metabolite production. Depletion of nitrogen resulted an increase in the level of plasmin inhibitor in suspension cultures of *Scopolia japonica* (Misawa et al., 1975) and solasodine from *Solanum lacinatum* cultures (Chandler & Dodds, 1983). However, there are some exceptions, wherein, higher levels of nitrogen resulted in increased product formation. For example, in *Lithospermum erythrorhizon* cultures,

the production of 1,4-naphthaquinone was increased with increase in nitrate level to above 67 mM and upto 104 mM (Mizukami *et al.* 1978) and similar increase in shikonin production was reported when the nitrate level was incrased from 3.3 to 6.7 mM (Fujita *et al.*, 1981).

Apart from changes in the levels of these nutrients, the source and ratio of NO_3 to NH_4 were found to influence product formation. For example, in *N.tabacum* cultures, ubiquinone formation was enhanced when NH_4^+ was the sole nitrogen source (Ikeda *et al.* 1976). On the contrary, *Lithospermum erythrorhizon,* the cells were not able to tolerate NH4 and the production of shikonin was completely inhibited when NH4 was present in the medium (Fujita *et al.* 1981). Variation in product formation due to altered ratio was also reported in *Anchusa officinalis* cultures (De-Eknamkul & Ellis, 1985). In most cases, an inverse relation with growth was noticed during nitrate stress.

In the present investigation, it was observed that when the actively growing cells were transferred to NO_3 free medium, a positive influence was noticed and about 31% yield increase could be achieved (Fig.6.2.2), whereas half the level of nitrate concentration was led to 46% yield increase. This observation strengthened the fact that *S.rhombifolia* cultures required both ammoniacal and nitrate nitrogen for growth and ephedrine production. Further, it was envisaged that when the nitrogen was depleted in the medium the rate of protein synthesis might slow down and the unutilised carbohydrates could be diverted to secondary metabolite synthesis.

Phosphate regimes trigger the production of various secondary metabolites including antibiotics and alkaloids in microbial cultures (Drew & Demain, 1977). Influence of phosphate in plant cell culture system has also been reported (Mantell & Smith, 1984; Dicosmo & Towers, 1984). Cultured cells of *Catharanthus roseus* grown in media depleted of inorganic phosphate showed increased synthesis of indole alkaloids (Knobloch *et al.* 1982) whereas, in tobacco cultures, decreased phosphate level led to reduction in the time of nicotine accumulation (Knobloch & Berlin, 1980). The optimal phosphate concentration for shikonin synthesis in *L.erythrorhizon* was half the optimal concentration for growth (Fujita *et al.* 1981).

In the present investigation, medium without phosphate effected 2.6 fold increase in ephedrine yield while medium with half the conc. of phosphate promoted 1.73 fold increase in ephedrine production.

The regulatory role of phosphate in secondary metabolism in plant cell culture systems is well understood. In tobacco cultures while high phosphate concentration resulted in a decrease in phenylalanine ammonia lyase (PAL) enzyme activity, phosphate depletion led to marked increase in the activity of PAL followed by increase in the biosynthesis of *cinnamoyl putrescenes* (Knobloch, 1982). It was also envisaged that cells may take up phosphate within two days from inoculation into fresh medium and phosphate could be stored (like nitrate) in vacuoles from where it was depleted during growth. So no secondary metabolite formation could occur as long as phosphate was present in the vacuole, suggesting a possible regulatory role for phosphate (Wray *et al.* 1983).

However, among the influence of various stress factors studied, phosphate stress was observed as most effective for ephedrine production in *S.rhombifolia* suspension cultures and phosphate was chosen as the limiting nutrient. Further, a major percentage of the total ephedrine produced was leached into the medium (42%) under phosphate stress, which is highly advantageous in plant cell culture system. The regulatory role of phosphates in enhancing ephedrine production and the mechanism by which the alkaloid leaks abundantly into the medium needs to be elucidated.

6.3 Yield enhancement by elicitation of cell cultures of *S.rhombifolia* Linn.

6.3.1 Introduction

Elicitors can be strictly defined as compounds of biological origin involved in plant/microbial interactions (biotic elicitors). This definition has, however, been broadened to include abiotic elicitors which include UV light (Jones, 1984), pH changes (Wink, 1985), osmotic stress (Dudge & Morris, 1986), wounding (Wink, 1984) and heavy metal ions (Watson & Brooks, 1984) which have also been shown to induce changes in plant secondary metabolism. Treatment of plants/tissue cultures with elicitors frequently leads to the accumulation of antimicrobial compounds (phytoalexins) which are thought to play a role in disease resistance (Bailey & Mansfield, 1982) and which are not present or present in very low concentration in untreated plants or cultures. In a few instances, the chemical composition of the

elicitor molecule is known and they have been shown to consist of a diverse group of compounds including oligosaccharides and polysaccharides.

The recent development of elicitation of plant cell cultures has opened a new avenue for the enhanced production of secondary metabolites (Dicosmo & Misawa, 1985). Preparations ranging from complex culture homogenates to highly purified fractions have been employed to initiate defence response leading to phytoalexin accumulation.

In the present investigation, the influence of some fungal elicitors and abiotic elicitors on the enhancement of ephedrine production in cell suspension cultures of *S.rhombifolia* Linn. ssp. *retusa (*Linn.*)* Borssum was determined.

6.3.2 Materials and methods

6.3.2.1 Callus induction and maintenance

The callus culture of *S.rhombifolia* was initiated and maintained as described under the section 3.2.3.

6.3.2.2 Initiation of suspension culture and maintenance

The suspension culture was initiated and maintained as described under section 5.2.3.

6.3.2.3 Growth measurement

The growth of the cells was measured as described under section 5.2.8.

6.3.2.4 Extraction procedure

The ephedrine was extracted from the cells as well as from the medium as described under section 5.2.9.

6.3.2.5 Analysis of ephedrine content

The ephedrine content was analysed as described under section 3.2.6.

6.3.2.6 Source of fungal elicitors

The fungal elicitors used for elicitation studies were *Pythium aphanidermatum* and *Rhizoctonia solanii*. The pure cultures were obtained from the Department of Plant Pathology and Microbiology, College of Horticulture, Kerala Agricultural University, Thrissur.

6.3.2.7 Elicitor preparation

Both the fungi were grown in 250 ml flasks containing 50 ml sterile potato dextrose broth (HI Media, India) at pH 3.5 (the pH was adjusted using sterile 10% tartaric acid after autoclaving the medium). The cultures were incubated at room temperature (30°C). At the stationary phase (after 20 days), the flasks with the cultures were autoclaved and the fungal mat was separated by filtration. The mat was then washed several times with distilled water and an aqueous extract was made by homogenizing in a mortar and pestle using acid washed neutralised sand. This extract was filtered through musclin cloth and the volume was made equal to that of filtrate with distilled water. The extracts and filtrates were sterilized by autoclaving before use.

6.3.2.8 Fungal elicitor concentration

Effect of fungal elicitors on ephedrine production was tested using Media Filtrates (MF) and Mycelial Extracts (ME) at two concentrations (5% and 10% v/v). The elicitors were added to the ten day old cultures and ephedrine production was assessed at 4 day intervals (4^{th} and 8^{th} day after elicitor treatment). The control flasks received an equal amount of sterile distilled water.

6.3.2.9 Abiotic elicitors

The trace elements present in the basal medium viz., calcium ($CaCl_2.2H_2O$), manganese ($MnSO_4.H_2O$), zinc ($ZnSO_4.7H_2O$), copper ($CuSO_4.7H_2O$), iron ($FeSO_4.7H_2O$) and cobalt ($CoCl_2.2H_2O$) were tried as abiotic elicitors.

Twenty fold increase of the concentration present in the basal media was used for elicitation purpose. Ten day old cultures were centrifuged and the cells were transferred aseptically into the sterile medium containing abiotic elicitors to study their influence on ephedrine production. The elicitation effect of these abiotic elicitors was assessed by harvesting the cells at 4 day intervals (4^{th} day and 8^{th} day after elicitor treatment) and analysing the ephedrine content.

6.3.3 Results

Actively growing cell suspension cultures were subjected to elicitation treatment on the assumption that the cells at this stage will be very active physiologically and hence may be more suitable to be influenced by elicitor treatment.

The elicitation was monitored at 4 days interval after elicitation treatment (4^{th} and 8^{th} day).

6.3.3.1 Influence of *P.aphanidermatum* elicitors

From the data presented in Fig.6.3.1, it is evident that Mycelial Extract (ME) of *P.aphaniderenatum* was superior to MF (Media Filtrate), with respect to alkaloid production. An increase of 2.65 fold ephedrine was recorded with 5% ME treatment (1.98 mg/100 ml culture as against 0.75 mg/100 ml culture of control). When the concentration of ME elicitor was increased, a gradual reduction in the total yield of ephedrine was noted. The maximum yield was obtained after a contact time of 4 days with ME elicitors and the yield decreased when the incubation period was increased to 8 days after treatment.

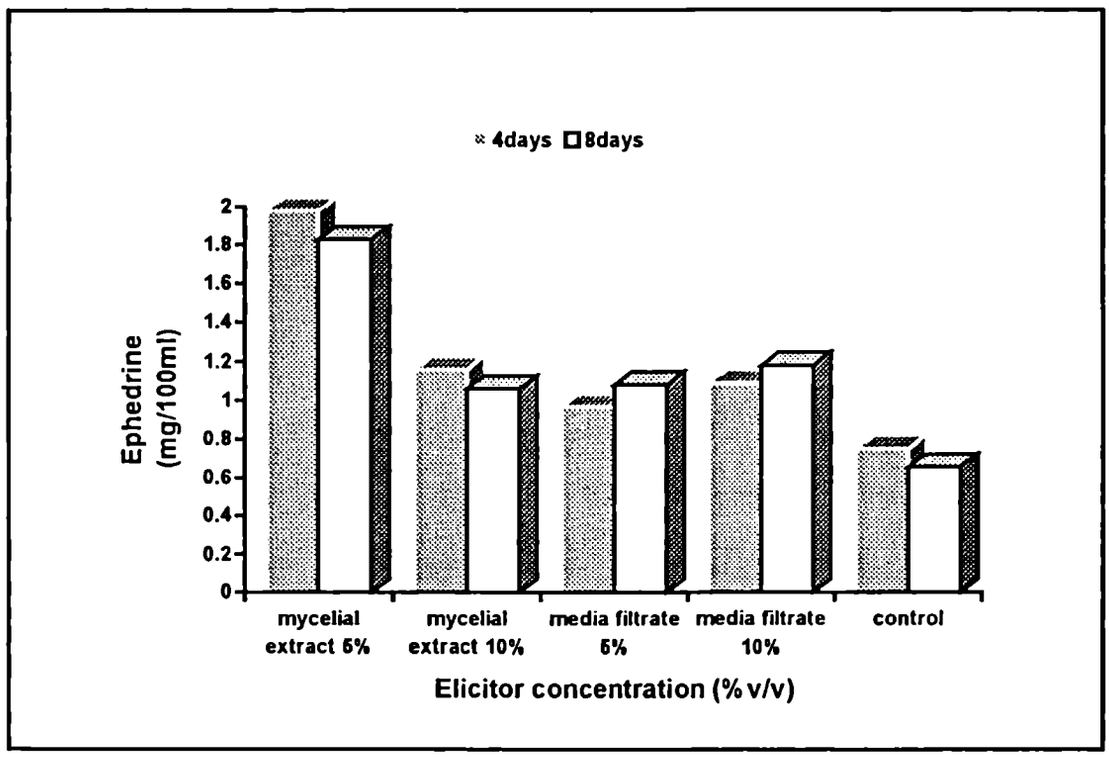
Compared to mycelial extract, the MF elicitor effected only less response. MF at 10% (v/v) level could achieve 1.56 fold increase. Unlike ME, the Media Filtrate required a higher concentration for positive effect. Further, the contact time required for elicitation was also more.

6.3.3.2 Influence of *Rhizoctonia solanii* elicitors

Both mycelial extract (ME) and media filtrate (MF) elicitors of *R.solanii* effected a positive influence on enhancement of ephedrine yield (Fig.6.3.2), though the foldwise final yield was less compared to that of *P.aphanidermatum* (1.66 mg/100 ml culture as against 1.98 mg/100 ml culture). Mycelial Extract was more effective than Media Filtrate as in the case of *P.aphanidermatum*. ME at 5% (v/v) level could

Fig 6.3.1

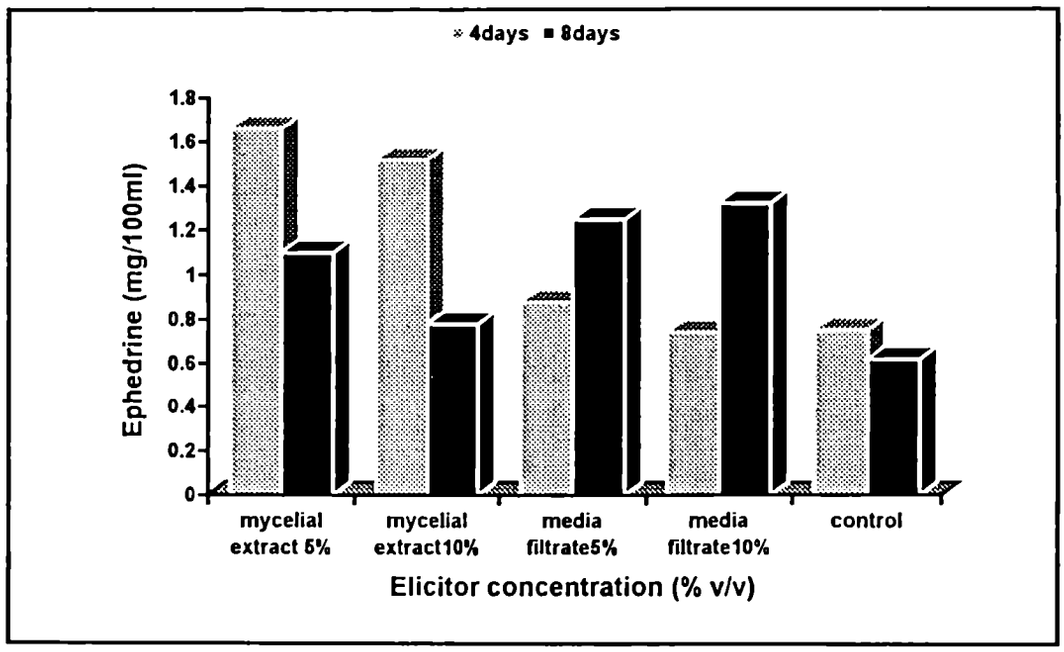
Ephedrine production in cell culture of *S.rhombifolia* under the influence of *P.aphanidermatum* elicitor



Data indicate average of 5 replicates. Cultures were incubated at 25±2^{0}C under 1000 lux light intensity on a rotary shaker at 90rpm. Elicitors were added to the actively growing cultures on 10th day of culture and production was assessed after 4 & 8 days of further incubation.

Fig 6.3.2

Ephedrine production in cell cultyres of *S.rhombifolia* under the influence of *R.solanii* elicitor



Data indicate average of 5 replicates. Cultures were incubated at 25±2°C under 1000 lux light intensity on a rotary shaker at 90rpm. Elicitors were added to the actively growing cultures on 10th day of culture and production was assessed after 4 &8 days of further incubation.

promote 2.21 fold increase in ephedrine production. When the concentration was increased to 10%, the alkaloid yield decreased. Four days contact time was effective than 8 days. But when MF was used, a higher concentration of 10% and a long incubation period of 8 days were more effective.

6.3.3.3 Influence of abiotic elicitors

Among the various abiotic elicitors tried, calcium showed the maximum positive influence (1.76 fold increase) followed by Mn (1.52 fold) and Zn (1.36 fold) (Fig.6.3.3), whereas Cu, Fe & Co showed negative influence. The influence was more pronounced on the 4^{th} day after elicitation treatment rather than on the 8^{th} day.

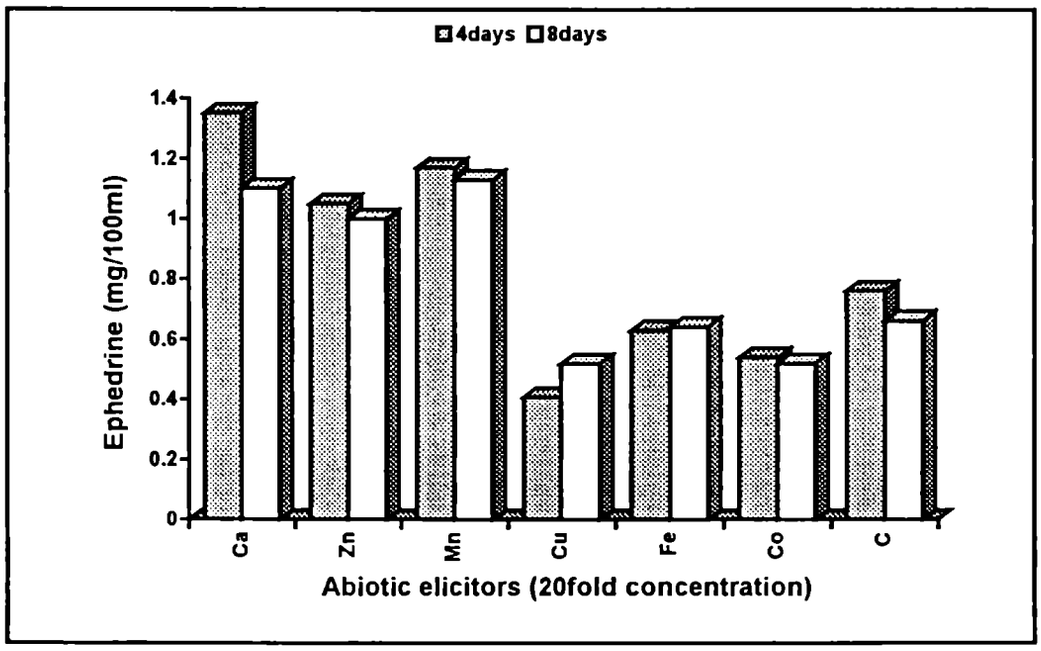
6.3.4 Discussion

With respect to secondary metabolite formation in cell cultures, elicitor effect must result in increased production of the desired product. Altered patterns of product accumulation in response to elicitation has been reported (Mukundan & Hjortso, 1990; Ramakrishna *et al.* 1993; Johnson 1993; Rajendran 1994). The effect of any elicitor (biotic or abiotic) is dependent on a number of factors which may interact – specificity of elicitor, elicitor concentration, the duration of treatment and the growth stage of the culture (Holden *et al.* 1988).

The elicitor chosen, if biotic, the best is the one normally derived from a microorganism pathogenic to the plant species of interest (Darvill & Albersheim, 1984; Ebel *et al.* 1984). The preparation derived from non-pathogenic microorganisms have also been successfully employed (Funk *et al.* 1987). In the

Fig 6.3.3

Ephedrine production in cell cultures of *S.rhombifolia* under the influence of abiotic elicitors



Data indicate average of 5 replicates. Cultures were incubated at 25 ± 2^{0}C under 1000 lux light intensity on a rotary shaker at 90rpm. Actively growing cells, on 10th day of culture were transfered to fresh sterile medium containing abiotic elicitors and production was assessed after 4 & 8 days of further incubation.

present investigation, since no pathogenic organism of *S.rhombifolia* was reported so far, the choice of micro organism was done based on the positive results reported in the literature (Endress, 1994), and also on ready availability of microbial cultures.

The response of cell cultures to elicitation may vary at different stages of growth cycle. Cultures are routinely elicited during late exponential phase of cell growth. Eilert *et al.* (1986) have demonstrated that older cultures (10 d) produced more indole alkaloids than young (5d) cultures under the influence of elicitors. Hence cultures at late exponential phase (10 day) were used for elicitation treatment in the present study.

Both the fungal elicitors (*Pythium aphanidermatum* and *Rhizoctonia solanii* influenced the ephedrine production positively in the cell cultures of *S.rhombifolia*. In both the cases, mycelial extract was more effective than media filtrates (2.65 fold for *P.aphanidermatum* and 2.21 fold for *R.solanii*). Regarding the elicitor concentration, 5% and 10% levels were best for ME and MF respectively. Further, the ME treatment required only less contact time (4d) for maximum yield enhancement as against 8d for MF treatments. This kind of variation in incubation time and concentration of elicitor requirement has been reported in other systems (Funk *et al.* 1987; Bonhoff & Griesbach 1988; Buitelaar *et al.* 1992).

There are some encouraging reports of the use of metal ions to induce or enhance the production of pharmaceuticals in cell suspension culture system. Fujita *et al.* (1982) reported that when the copper(II) concentration in the production

medium of *L.erythrorhizon* was raised to a level of 30 times than the normal, there was a dramatic stimulation in the production of shikonin. Enhanced level of Vanadium(II) sulphate resulted in the accumulation of indole alkaloids in *C.roseus* (Threlfall & Whitehead, 1988a&b). In the present investigation, 20 fold increase of Ca, Mn and Zn resulted in enhanced yield of ephedrine.

The use of abiotic elicitors offers many advantages over the biotic counterparts—their ready availability, relative cheapness, ease of use and the fact that they are chemically defined. Eventhough it is difficult to explain the mechanism of their effect on the secondary metabolism of plant cultures, the fact remains that they achieve the desired results in some cases. Hence there is a lot of scope for further research in this field.

The results presented here are the first of this kind on the elicitation of the alkaloid ephedrine in cell culture system.

6.4 Yield enhancement studies by the use of permeabilization agents

6.4.1 Introduction

One of the major problems associated with plant cell culture system is that the products formed by cultured plant cells are stored in the vacuoles and the release of these products is difficult without reducing the cell viability. In order to extract the product without destroying the cells and to enable continuous cultivation system, the

products formed has to be released into the medium. The products from vacuoles have to penetrate two membrane barriers; plasma membrane and tonoplast, so as to get released into the medium.

Cell permeabilization depends on the *de novo* formation of pores in one or more of membrane systems of plant cell, which enables the "unnatural" passage of various molecules into or out from the cell. On one hand, there have been permanently permeabilized cell systems that use the cells as "dead" catalytic sites for enzyme reactions (Felix & Mosbach 1982), while on the other hand there have been attempts to permeabilize the cells only transiently, to enable the cells to remain "live" and yet have short time periods when rates of mass transfer of substrates and metabolites into or out from the cells can be increased (Parr *et al.* 1984). Cells so treated can be repeatedly subjected to permeabilization, thus permitting the maximum use of cells and avoiding a "destructive" harvest to obtain the cell product, but so far long term permeabilization treatments with chemical agents have not been successful.

Several attempts were made for the non-destructive cell permeabilization treatments for the enhanced release of plant secondary metabolites (Kilby & Hunter, 1986; Kilby 1987; Schmidt *et al.* 1989). But only a few techniques utilizing dimethyl sulphoxide (Bordelius & Nilsson, 1983; Schmidt *et al.* 1989) have been reported to be successful in a very limited number of systems for product release and maintenance of cell viability. Park and Martinez (1994) used a new technique for retaining the cell viability in permeabilization using DMSO which involved the use of preconditioning with 0.1% DMSO before the cells were permeabilized by higher concentrations of

DMSO. Apart from organic solvents, some polysaccharides such as chitosan (Young & Kauss, 1983; Knorr & Teutonico, 1986) and laminarin (Johnson, 1993) were also reported to be useful as permeabilizing agents of plant cell culture systems.

In this study, the organic solvent DMSO and the natural polysaccharides such as chitosan and liminarin were tried to permeabilize the membrane system of *S.rhombifolia* cell cultures without seriously affecting the cell viability for extraction of the alkaloid without destroying the cells and facilitating reuse.

6.4.2 Materials and methods

6.4.2.1 Source of plant material

The source of plant material was as described under section 3.2.2.

6.4.2.2 Callus induction and maintenance

The callus culture of *S.rhombifolia* was initiated and maintained as described under section 3.2.3.

6.4.2.3 Initiation and maintenance of suspension culture

The suspension culture was initiated and maintained as detailed under section 5.2.3.

6.4.2.4 Cell permeabilization using DMSO '

Ten day old, actively growing, cells were harvested by centrifugation at 5000 rpm for 10 minutes (Kubota, Japan), and treated with 3 different concentrations of filter sterilized DMSO (0.1%, 0.5% and 1.0% v/v) in sterile medium for 30 minutes and incubated in an orbital shaker (90 rpm) at 25±2°C, under 1000 lux illumination.

After treatment, the cells were separated by centrifugation (5000 rpm for 10 minutes), washed repeatedly with sterile distilled water, made free of organic solvents, transferred to the medium and incubated in an orbital shaker (90 rpm), at 25±2°C, under 1000 lux light intensity for 8 days. The ephedrine production in the medium and biomass were estimated after 4 and 8 days of incubation as detailed under section 5.2.10.

6.4.2.5 Cell permeabilization using chitosan

Chitosan was dissolved in 0.1N acetic acid, pH adjusted to 5.5 with 1N NaOH and filter sterilized. Three different concentrations (5 μg ml^{-1}, 7.5 μg ml^{-1} and 10 μg ml^{-1}) were added aseptically to the actively growing ten day old cultures of *S.rhombifolia* and incubated in an orbital shaker (90 rpm) at 25±2°C under 1000 lux light intensity for 8 days. The ephedrine production was estimated both in cells and medium after 4 days and 8 days of permeabilization treatment as detailed under section 5.2.10.

6.4.2.6 Cell permeabilization using laminarin

Aqueous solution of laminarin was filter sterilized and added at two different concentrations (10 μg ml^{-1} and 20μg ml^{-1}), aseptically, to the actively growing ten day old cultures of *S.rhombifolia* and incubated in an orbital shaker (90 rpm) under 1000 lux light intensity at 25±2°C for 8 days. The ephedrine production was estimated both in the cells and medium after 4 days and 8 days of permeabilization treatment as detailed under section 5.2.10.

6.4.2.7 Cell viability percentage

The cell viability percentage was assessed by dye exclusion technique using Evan's Blue (Gaff & Okong O'-Ogola, 1971).

6.4.3 Results

6.4.3.1 Cell permeabilization using DMSO

Treatment of cells with 0.1% DMSO for 30 minutes, effected release of 38.50% ephedrine into the medium (Table 6.4.1) which was about 50% more compared to the untreated control, which released only 25.71%. On increase in the concentration of DMSO to 0.5% and 1.0%, the release was enhanced to 41.30% and 50.0% respectively. At 0.1% concentration of DMSO, the cell viability percentage was 71.0 compared to 84.0 in the untreated control. But, when the concentration was increased to 0.5% and 1.0%, the cell viability percentage got reduced to 54.2 and 38.6 respectively.

6.4.3.2 Cell permeabilization using chitosan

Addition of chitosan at 5 $\mu g\ ml^{-1}$ resulted in 56.2% release of ephedrine (Table 6.4.2) whereas for 7.5 and 10.0 $\mu g\ ml^{-1}$ concentration, the percentage release got reduced to 48.4 and 35.0 respectively. Further, when chitosan was used as the permeabilizing agent, there was a reduction in cell viability percentage. The cell viability percentage was above 60 for concentration upto 7.5 $\mu g\ ml^{-1}$. However, the cell viability was further reduced to 55.2% when the concentration was increased to

Table 6.4.1

Influence of DMSO on cell permeabilization *of* *S.rhombifolia* Linn. cell cultures

DMSO concentration (% v/v)	Period of incubation (days)	Biomass (gfw/100 ml culture)	Ephedrine (mg/100 ml culture)			Percentage release	Cell viability %
			Cell	Medium	Total		
0	4	15.7	0.52	0.18	0.70	25.71	84.0
	8	17.9	0.55	0.18	0.73	24.70	81.1
0.1	4	14.7	0.40	0.25	0.65	38.50	71.0
	8	14.2	0.40	0.24	0.64	37.64	64.4
0.5	4	14.2	0.37	0.26	0.63	41.30	54.2
	8	14.2	0.37	0.26	0.63	41.30	51.7
1.0	4	13.8	0.31	0.31	0.62	50.00	38.6
	8	13.8	0.30	0.30	0.60	50.00	36.3

The data represent average of 5 replications. The cultures were incubated at 25±2°C in an orbital shaker (90 rpm) under 1000 lux light intensity.

Table 6.4.2

Influence of chitosan on cell permeabilization of *S.rhombifolia* Linn. cell cultures

Chitosan $\mu g\ ml^{-1}$	Period of incubation (days)	Biomass (gfw/100 ml culture)	Ephedrine (mg/100 ml culture)			Percentage release	Cell viability %
			Cell	Medium	Total		
0	4	18.0	0.51	0.16	0.67	24.3	82.9
	8	18.9	0.55	0.18	0.73	24.7	81.3
5	4	16.8	0.28	0.36	0.64	56.2	65.4
	8	18.1	0.31	0.30	0.61	49.2	68.2
7.5	4	16.6	0.32	0.30	0.62	48.4	62.9
	8	17.6	0.38	0.27	0.65	41.5	60.2
10.0	4	17.4	0.39	0.21	0.60	35.0	55.2
	8	17.1	0.43	0.22	0.65	30.8	50.0

The data represent average of 5 replications. The cultures were incubated at 25±2°C in an orbital shaker (90 rpm) under 1000 lux light intensity.

10 μg ml^{-1}. Moreover, there was a decrease in percentage release, when the incubation period was increased to 8 days after permeabilization treatment.

6.4.3.3 Cell permeabilization using laminarin

Addition of 20 μg ml^{-1} laminarin resulted in 50% release of products into the medium with 60% cell viability (Table 6.4.3), whereas addition of 10 μg ml^{-1} not only resulted in 43.8% release, but also retained a very high cell viability percentage of 70.9. There was no further increase in the release of products when incubated for 8 days after permeabilization treatment when compared to 4 days of incubation.

6.4.4 Discussion

Among the three permeabilizing agents tried in the present study, permeabilization using chitosan (5 μg ml^{-1}) was observed as ideal treatment for *S.rhombifolia* cell culture, as it enhanced the release of alkaloids to 56.2% from 24.3% in the untreated control and retained about 65.4% cell viability. When the organic solvent DMSO was used, 50% release of the product was achieved with 1.0% DMSO concentration but the cell viability was reduced to below 40%.

Chitosan (β-1,4-linked glucosamine) is a major component of the fungal cell wall (Bartnicki-Garcia 1970) and is formed by enzymatic deacetylation of chitin (Araki & Ito, 1975). This polycationic polymer appears to be involved as a regulatory molecule in a number of different biological systems. Plant cell cultures have been used to study the effect of chitosan on plant cell metabolism. The formation of

Table 6.4.3

Influence of laminarin on cell permeabilization of *S.rhombifolia* Linn. cell cultures

Laminarin µgm l^{-1}	Period of incubation (days)	Biomass (gfw/100 ml culture)	Ephedrine (mg/100 ml culture)			Percentage release	Cell viability %
			Cell	Medium	Total		
0	4	19.6	0.51	0.15	0.66	22.7	83.6
	8	19.9	0.53	0.14	0.67	20.9	80.9
10	4	19.5	0.36	0.28	0.64	43.8	70.9
	8	18.7	0.43	0.29	0.72	40.3	70.1
20	4	19.1	0.29	0.29	0.58	50.0	60.0
	8	18.8	0.39	0.32	0.71	45.0	60.3

The data represent average of 5 replications. The cultures were incubated at 25±2°C in an orbital shaker (90 rpm) under 1000 lux light intensity.

phytoalexins, glyceollin and acridone epoxides were studied in *Glycine max* (Kohle *et al.* 1984) and Ruta graveolens (Eilert *et al.* 1984), respectively. Chitosan is reported to bind to polygalactouronate of the cell wall, thereby inducing permeability of low molecular weight compounds (Young *et al.* 1982).

Beaumont *et al.* (1989) showed that different plant cell suspensions treated with chitosan exhibited normal viability and enhanced protein release in the medium compared with control free cell suspensions. Similarly in the present investigation also, an enhanced release of ephedrine into the medium (56.2%) without affecting much the cell viability (65.4%) could be achieved. However, detailed studies are needed to investigate the impact of chitosan on cell viability percentage.

Further, as our product of interest being a pharmaceutical compound, use of natural polysaccharides such as chitosan and laminarin are preferred rather than toxic chemicals like DMSO.

6.5 Studies on yield enhancement by precursor feeding

6.5.1 Introduction

Plant cell cultures perform various transformation reactions of exogenously supplied compounds (Reinhard & Alferman, 1980). Bioconversions may be exploited in the synthesis of products which are normally extracted from the plant or in the formation of novel products (Pars, 1992). In a number of experimental systems it was shown that the enzymes of secondary metabolites did not work at their maximum rates because the concentration of precursors, co-substrates and other necessary

intracellular compounds were too low (Luckner, 1990). Therefore, an increase in the intracellular concentrations of precursors and other rate limiting compounds by addition to the medium may result in increased rates of secondary metabolite synthesis.

The biotransformation reactions are usually intracellular. The permeable substrates are taken across the cell wall, through enzymatic compartments, and the reaction products subsequently released extracellularly (Johnson, 1993). The projected potentials of biotransformation lie in the conversion of less expensive bulk products to value added, more bioactive and low volume products. Precursor feeding has been used successfully to improve the alkaloid yield in a number of plant cell cultures. Zenk *et al.* (1977) achieved a 3 fold increase in alkaloid production by feeding L.tryptophan to *Catharanthus roseus* cultures. Hay *et al.* (1986) reported a 5 fold improvement in quinine and quinidine yield from root organ cultures of *Cinchona ledgeriana* by feeding *L.tryptophan*.

In the present study, the influence of precursor feeding on ephedrine production in *S.rhombifolia* cell culture system was evaluated using phenylalanine and methionine.

6.5.2 Materials and methods

6.5.2.1 Source of plant material

The source of plant material was as described under section 3.2.2.

6.5.2.2 Callus induction and maintenance

The callus culture of *S.rhombifolia* was initiated and maintained as described under section 3.2.3.

6.5.2.3 Initiation and maintenance of suspension culture

The suspension culture was initiated and maintained as detailed under section 5.2.3.

6.5.2.4 Influence of precursor feeding on ephedrine production in *S.rhombifolia* cell cultures

The strategy adopted for precursor feeding was that the precursor was added to the ten day old actively growing cultures and the ephedrine production was assessed on 14^{th} and 18^{th} day, as detailed under section 5.2.10.

6.5.2.5 Influence of phenylalanine on ephedrine production

Influence of phenylalanine on ephedrine production was studied using filter sterilized phenylalanine in double distilled water added at 3 levels (0.75 mM, 1.5 mM and 3.0 mM).

6.5.2.6 Influence of methionine on ephedrine production

Effect of methionine on ephedrine production was assessed using methionine at 3 levels (0.75 mM, 1.5 mM and 3.0 mM) prepared in double distilled water and filter sterilized using a millipore filter sterilizer assembly.

6.5.2.7 Culture conditions

The precursor treated cultures were incubated for 8 days on a rotary shaker (90 rpm) under 1000 lux light intensity at 25 ±2°C. MSSR-4 medium with 3% sucrose was used to culture the cell.

6.5.2.8 Growth measurement

The growth of the cells was measured as described under section 5.2.8.

6.5.2.9 Extraction procedure

The ephedrine was extracted from the cells as well as from the medium as described under section 5.2.9.

6.5.2.10 Analysis of ephedrine content

The ephedrine content was analysed as described under section 3.2.6.

6.5.3 Results

6.5.3.1 Influence of L-phenyl alanine on ephedrine production

Addition of 0.75 mM L-phenyl alanine promoted an increase of 1.71 fold ephedrine yield (Table 6.5.1) on 18^{th} day of culture (1.40 mg/100 ml culture compared to 0.82 mg/100 ml culture). When the level of the precursor was raised to 1.5 mM and 3.0 mM, the increase of ephedrine yield was comparatively less (1.52 and 1.50 fold respectively). The increase in ephedrine yield after 4 days of precursor feeding was less compared to 8 days of incubation.

Table 6.5.1

Influence of L.phenylalanine on ephedrine production in *S.rhombifolia* Linn. Cell cultures

Precursor	Biomass (gfw/100 ml culture)	Ephedrine (mg/100 ml culture)			Foldwise increase
		Cell	Medium	Total	
Control	18.95	0.60	0.22	0.82	--
PA.1-4	20.30	1.06	0.31	1.37	1.67
PA.2-4	20.25	0.91	0.28	1.19	1.45
PA.3-4	20.00	0.89	0.27	1.16	1.41
PA.1-8	20.25	1.09	0.31	1.40	1.71
PA.2.8	20.25	0.97	0.28	1.25	1.52
PA.3-8	20.25	0.95	0.28	1.23	1.50

PA1 – Phenylanine 0.75 mM, PA.2 – Phenylalanine 1.5 mM, PA.3 – Phenylalanine 3.0 mM.

4 - Four days incubation after precursor feeding, 8 - Eight days incubation after precursor feeding. The precursor fed cultures were incubated in an orbital shaker (90 rpm) at 25±2°C under 1000 lux light intensity.

6.5.3.2 Influence of L-methionine on ephedrine production

Addition of 1.5 mM L-methionine resulted in an increase of 1.63 fold ephedrine yield (1.29 mg/100 ml culture against 0.79 mg/100 ml culture) after 4 days of precursor feeding (Table 6.5.2). Though there was an increased production of ephedrine for 0.75 mM and 3.0 mm levels of methionine (1.39 fold and 1.34 fold respectively), the foldwise increase was less compared to 1.5 mM level. Further, incubation upto 8 days after precursor feeding did not enhance ephedrine yield.

6.5.4 Discussion

The addition of phenylalanine in the cultures of *Datura* (Sairam & Khanna, 1971) and *Lithospermum* (Mizukami *et al.* 1978) caused a two to three fold increase in the amounts of secondary metabolites whereas the use of *tryptophan* (Valiky, 1972) in *Phaseolus vulgaris* cell suspension produced two new alkaloids harman and norharman. Ramawat & Arya (1979b) reported the addition of 100 mg l^{-1} phenyl alanine in callus cultures of *E.gerardiana*, resulted in 3 fold increase of ephedrine yield whereas when the level of precursor was raised to 400 mg l^{-1}, only 2 fold increase in ephedrine yield was effected. They also reported that when the callus cultures of *E.gerardiana* were fed with the precursor methionine (100 mg l^{-1}), more than 2 fold increase in ephedrine yield could be achieved. Similarly, when the level was raised to 400 mg l^{-1}, the foldwise increase was comparatively less. Further, Yamasaki *et al.* (1973) has reported that precursors, phenylalanine and methionine had an important role in ephedrine biosynthesis, and suggested the possible pathways for ephedrine synthesis in plants. Phenylalanine is directly incorporated into the

Table 6.5.2

Effect of methionine on ephedrine production in *S.rhombifolia* Linn. cell cultures

Precursor	Biomass (gfw/100 ml culture)	Ephedrine (mg/100 ml culture)			Foldwise increase
		Cell	Medium	Total	
Control	18.9	0.59	0.22	0.79	--
M.1-4	19.9	0.86	0.24	1.10	1.39
M.2-4	20.4	0.99	0.30	1.29	1.63
M.3-4	19.4	0.81	0.25	1.06	1.34
M-1-8	19.5	0.89	0.25	1.14	1.44
M.2-8	20.4	0.97	0.30	1.27	1.61
M-3-8	19.5	0.81	0.24	1.05	1.33

M.1 – Methionine 0.75 mM, M.2 – Methionine 1.5 mM, M.3 – Methionine 3.0 mM.

4 - Four days incubation after precursor feeding, 8 - Eight days incubation after precursor feeding. The precursor fed cultures were incubated in an orbital shaker (90 rpm) at 25±2°C under 1000 lux light intensity.

nitrogen of ephedrine and showed a profound effect on cultured tissues grown on phenylalanine supplemented medium while, methionine is partially incorporated in the biosynthesis of ephedrine.

In the present investigation, addition of the precursor L-phenylalanine resulted in 1.71 fold increase of ephedrine and methionine about 1.63 fold increase (Tables 6.5.1 and 6.5.2). The ephedrine production was maximum in the late logarithmic phase, when the protein synthesis and growth were ceased. During the latter period of culture, auxin and nitrogen might be exhausted in the medium and consequently protein synthesis and growth might be ceased, and these precursor aminoacids might have been used for ephedrine synthesis.

The concentration of precursors, the time of feeding and the incubation period after feeding, influence secondary product formation. In the present study, a lower concentration of 0.75 mM, L-phenyl alanine yielded maximum ephedrine compared to higher concentration of 1.5 mM and 3.0 mM level, (1.52 & 1.50 fold respectively). Further, the maximum increase was obtained after 8 days of incubation after precursor addition though the increase after 4 days of incubation and 8 days incubation was not significant. But when methionine was used as precursor, a higher concentration of 1.5 mM yielded maximum ephedrine and maximum increase was obtained 4 days after precursor feeding. The influence of phenylalanine on ephedrine production might be due to the fact that it might be directly incorporated into ephedrine biosynthesis unlike methionine which might have been partially incorporated into ephedrine biosynthesis as suggested by Yamasaki *et al.* (1973). In this study, as the precursors were

administered in the mid to late exponential phase when the growth and protein synthesis were ceased due to N exhaustion in the medium, the added precursors might be used for ephedrine production.

CHAPTER VII

Studies on ephedrine production by immobilized viable *Sida rhombifolia* cells

7.1 Introduction

Plant cells are relatively sensitive to changes in the environment and consequently only the mildest methods of immobilization need be employed. Of the various techniques available for immobilization, the entrapment technique is most appropriate for large sensitive plant cells (Brodelius, 1990). A number of researchers have suggested that calcium alginate is superior to other entrapping materials for plant cells (Brodelius, 1988; Williams & Mavituna, 1992). Hence in the present investigation, attempts were made to immobilize the *S.rhombifolia* cells by entrapment in calcium alginate gel and for evaluating the production profile of the immobilized cell system.

7.2 Materials and methods

7.2.1 Production medium for immobilized cell beads

Half MSSR-4 medium supplemented with 1 mg l^{-1} 1BA + 0.3 mg l^{-1} kin and 5 μg ml^{-1} chitosan as permeabilizing agent was used as the medium for incubating the immobilized cell beads of *S.rhombifolia* Linn. Further the precursor, phenyl alanine (100 mg l^{-1}) and the mycelial extract of the fungal elicitor *P.aphanidermatum* (5% level) were added to the medium to enhance the production of the alkaloid.

7.2.2 Preparation of immobilized viable cells

Ephedrine production by *S.rhombifolia* Linn. cells under immobilized condition was studied by subjecting the cells/small cell aggregates to gel entrapment in calcium alginate beads (Johnson, 1993). The gel entrapment procedure was initially standardized to obtain maximum production by *S.rhombifolia* Linn. cell culture system.

7.2.2.1 Preparation of cell suspension

Actively growing cells in the mid log phase (10 days old culture) were centrifuged at 10,000 rpm for 10 min. and the cells were transferred aseptically to half MS medium (10 g cells/30 ml medium).

7.2.2.2 Preparation of support material

Three grams of dry powder of sodium alginate (prior to optimization) was slowly added to 100 ml of half MS basal medium while being continuously stirred. The stirring was continued for a further period of 1-2 h in a magnetic stirrer until complete dissolution of the sodium alginate was effected. Sterilized the sodium alginate at 1.3 kg cm^{-2} for 20 min. and used for preparation of beads.

7.2.2.3 Preparation of beads

Under sterile conditions, the prepared cell slurry was mixed with the sodium alginate solution (3% w/v) at 1:1 ratio and mixed thoroughly using a sterile glass rod

to get a final concentration of 1.5%. The prepared sodium alginate cell slurry was extruded dropwise into an excess of sterilized half (MSSR-4) medium containing 50 mM $CaCl_2.2H_2O$ from a height of about 10 cm. The entrapped calcium alginate beads were maintained in half MSSR-4 medium with 50 mM $CaCl_2.2H_2O$ for 2h in an orbital shaker (90 rpm) at 25±2°C for curing. Washed the calcium alginate beads thoroughly with sterile half basal medium before transfer to production medium (half MSSR-4) and incubated on a rotary shaker (90 rpm) at 25±2°C under 1000 lux light intensity (16h photoperiod).

7.2.3 Activation of immobilized viable cells

The prepared beads were suspended in half MSSR-4 medium having a pH 5.5 in a conical flask and incubated in a rotary shaker (90 rpm) at 25±2°C under 1000 lux light intensity (16h photoperiod) for 48h (prior to optimiztion of activation time). The activated beads were then removed, washed twice with fresh production medium and used for further studies.

7.2.4 Assay of ephedrine

The ephedrine from both the medium as well as from the cells was estimated as mentioned below.

a) From the medium

The ephedrine from the medium was extracted and estimated as detailed under section 5.2.9.

b) From the immobilized cells

The immobilized cells were solubilized by the method of Jones and Veliky (1981). The beads were washed for 5 min. with distilled water, added with 0.1M potassium phosphate buffer (pH 7.5) and the mixture was agitated at 120 rpm for 3h on a rotary shaker. The cells were sedimented by centrifugation for 5 min. at 1000 rpm. After removing the cloudy supernatant with a pasteur pipette, the cells were washed, 3-4 times with phosphate buffer and distilled water. The resultant cells were recovered for ephedrine analysis which was carried out as detailed under section 5.2.9.

7.2.5 Optimization of factors that influence ephedrine production by immobilized viable cells of *S.rhombifolia*

Optimal concentration of support material, cell concentration in the beads, curing time of beads, activation time and retention time that could promote maximal production of ephedrine by the immobilized cells of *S.rhombifolia* were determined as outlined below.

7.2.5.1 Support concentration

The optimal support concentration required for the preparation of active and stable beads with maximum ephedrine production was determined using sodium alginate at different concentrations (final conc. at 1%, 1.5%, 2.0% and 2.5%). Immobilized viable cell beads were prepared as mentioned under section 7.2.2. Brodelius, 1990; Johnson, 1993). Ephedrine production was determined as mentioned under section 7.2.4.

7.2.5.2 Cell concentration in the beads

Immobilized cell beads were prepared using cells at different concentrations (1g to 2.5g cells in 6.5 ml of 1.5% sodium alginate), so that the final concentration of cells in the beads were 20%, 30%, 40% and 50% respectively. The cell concentration in the bead was determined by the method suggested by Brodelius (1990) as follows.

$$C = C_o \frac{\text{Weight of alginate + Cell suspension}}{\text{Weight of beads}}$$

C = Cell concentration in beads (w/w)

C_o = cell concentration in alginate cell mixture (w/w).

The ephedrine production was determined as mentioned under section 7.2.4.

7.2.5.3 Curing time

Optimum curing time for obtaining stable immobilized viable cell beads were determined by allowing the beads formed in the half MSSR-4 medium with 50 mM $CaCl_2.2H_2O$, to remain as such for varying periods of curing (0.5, 1, 1.5 2, 3 and 4h). After curing for various periods, the beads were washed with distilled water and the best curing time was assessed by testing the production of ephedrine, in the production medium.

7.2.5.4 Activation time

Optimal activation time was determined by incubating the immobilized cell beads in the production medium for varying time intervals (6, 12, 18, 24 and 48h),

immediately after preparation of the immobilized cell beads. The optimal activation time was assessed by testing the production of ephedrine in the production medium.

7.2.5.5 Retention time

Optimal retention time required for maximal production was determined by incubating the immobilized viable cell beads in the production medium for varying periods and estimating ephedrine production as mentioned under section 7.2.7.

7.2.5.6 Half life period

Half life period of immobilized viable cell beads was determined by incubating the immobilized viable cell beads in the production medium for 6 repeated cycles of 10 days each. The period taken for 50% reduction in the initial production potential of immobilized cell beads was taken as the half life period.

7.2.5.7 Comparison of the free cell system and immobilized system

Efficiency of immobilized viable plant cells for production of ephedrine was evaluated in comparison with that of free cells. For this purpose, an equal concentration of plant cells was used. Thus, 20 ml production medium (described under section 7.2.1) was inoculated with 1g free cells/1 g cells immobilized in calcium alginate and incubated for 14 days in an orbital shaker (90 rpm) at 25±2°C under 1000 lux light intensity (16h photoperiod).

7.3 Results

7.3.1 Optimization of process parameters

7.3.1.1 Support concentration

Results presented in fig.7.1 suggest that 1.5% alginate supported preparation of stable beads with maximum alkaloid production (540 μg/20 ml culture). At lower concentrations,the beads were not stable and were spongy whereas, at higher concentrations although the beads were very stable, the alkaloid yield got reduced (424 μg/20 ml culture).

7.3.1.2 Cell concentration in the beads

A cell concentration of 40% in the beads (Fig.7.2) (2.0 g cells in 6.5 ml of 1.5% sodium alginate) was most ideal for maximum production of ephedrine (596 μ g/20 ml culture) under immobilized condition.

7.3.1.3 Curing period

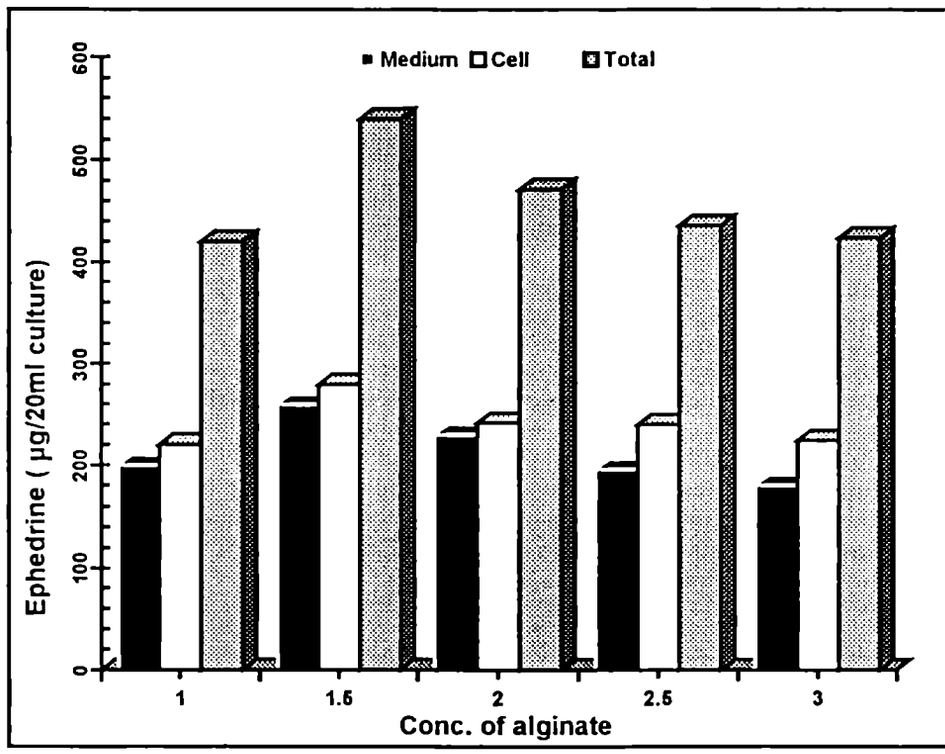
Results presented in Fig.7.3 suggest that a curing period of 2h is required to obtain maximum alkaloid production (612 μg/20 ml culture) under immobilized condition. Further increase in curing time did not enhance alkaloid production.

7.3.1.4 Activation time

The optimal activation time (Fig.7.4) for maximum ephedrine yield was 18h (636 μg/20 ml culture). When the activation time was further increased to 24h (620 μ

Fig 7.1

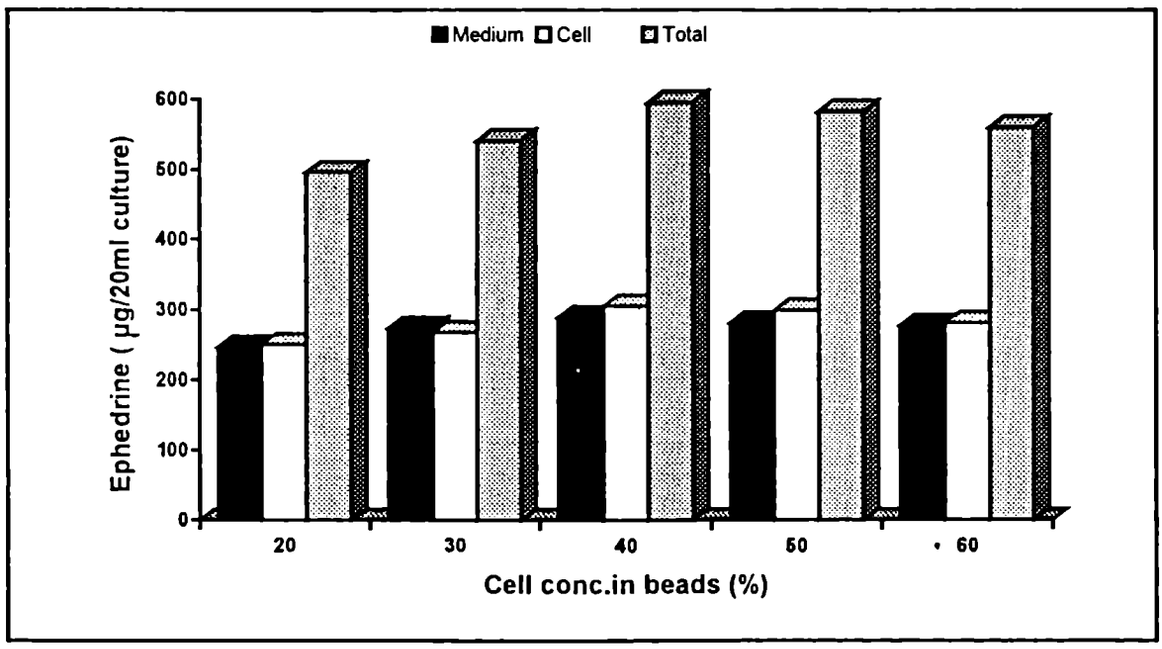
Optimisation of support concentration for the preparation of immobilized beads



1g cell was immobilized and incubated in 20ml medium for 10 days in an orbital shaker (90rpm) at 25±2^{0}C under 1000 lux light intensity (16h photoperiod).

Fig 7.2

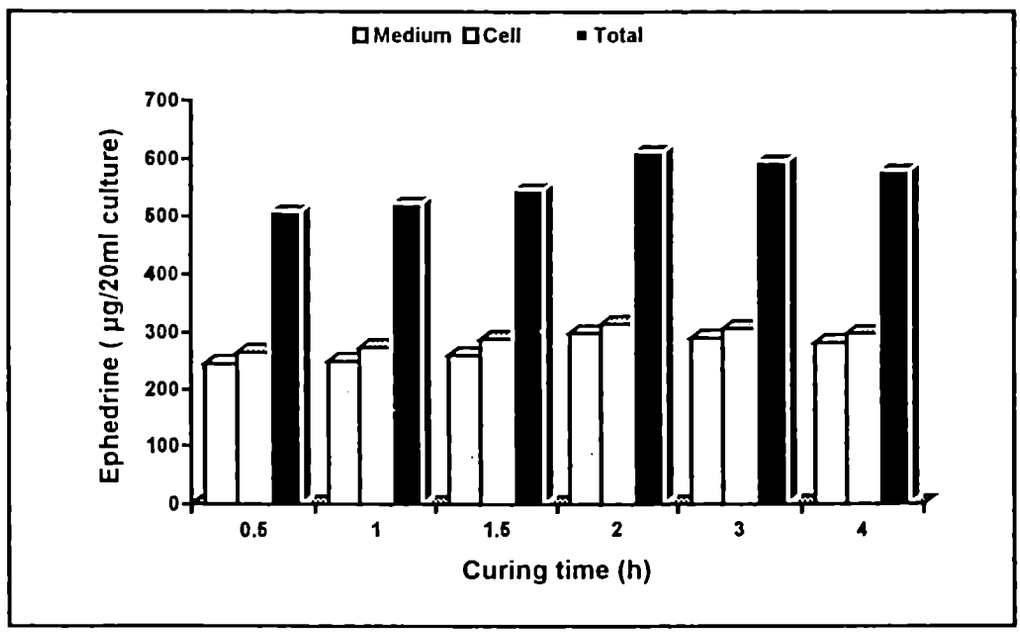
Optimisation of cell concentration for the preparation of immobilized beads



1g cell was immobilized and incubated in 20ml medium for 10 days in an orbital shaker 90rpm at 25±2^{0}C under 1000 lux light intensity (16h phoptoperiod).The data reperesent average of 5 replications.

Fig 7.3

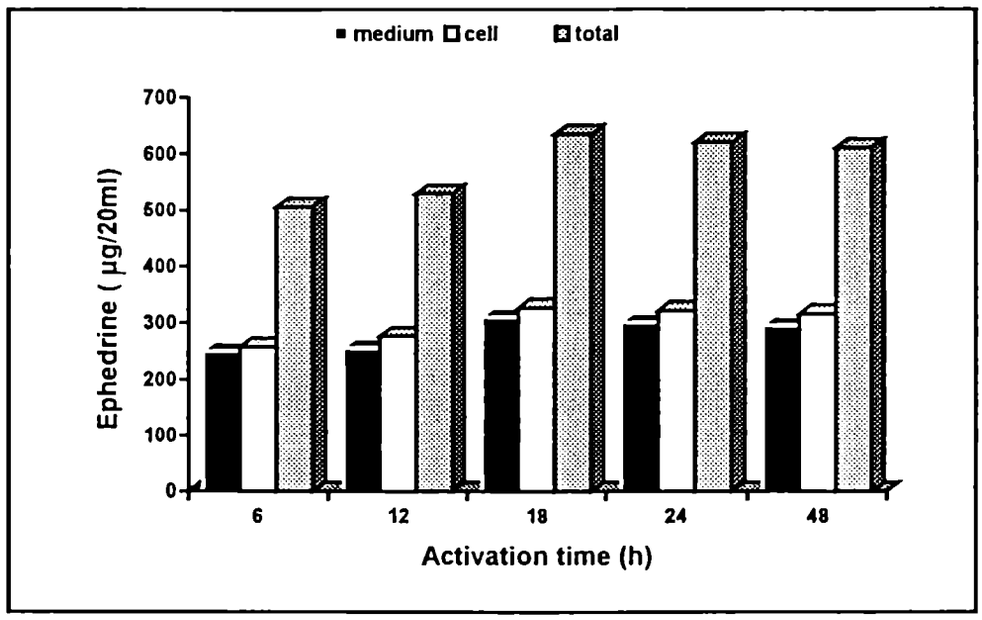
Optimisation of curing time for the preparation of immobilized beads



1g cell was immobilized and incubated 20ml medium for 10 days in an orbital shaker 90rpm at 25±2^{0}C under 1000 lux light intensity (16h phoptoperiod).The data reperesent average of 5 replications.

Fig 7.4

Optimisation of activation time of cell beads of *S.rhombifolia*



1g cell was immobilized and incubated in 20ml medium for 10 days in an orbital shaker (90rpm) at 25±2^{0}C under 1000 lux light intensity (16h photoperiod). The data represent average of 5 replicates.

g/20 ml culture) and 48h (610 μg/20 ml culture), there was only a marginal increase in ephedrine yield, unlike the rapid increase obtained with the 12h to 18h increase.

7.3.1.5 Retention time

The results presented in Fig.7.5 suggests that the optimal retention time for maximum alkaloid production was 10 days of incubation (662 μg/20 ml culture). The production remained almost static upto 15 days and then gradually declined.

7.3.1.6 Half life period

Half life period of the immobilized viable cells, after running 6 cycles of batch process of shake flask cultures was recorded as 3 cycles (Fig.7.6). After 3 cycles, the stability of the beads was very much reduced.

7.3.1.7 Comparison of free cell system and immobilized system

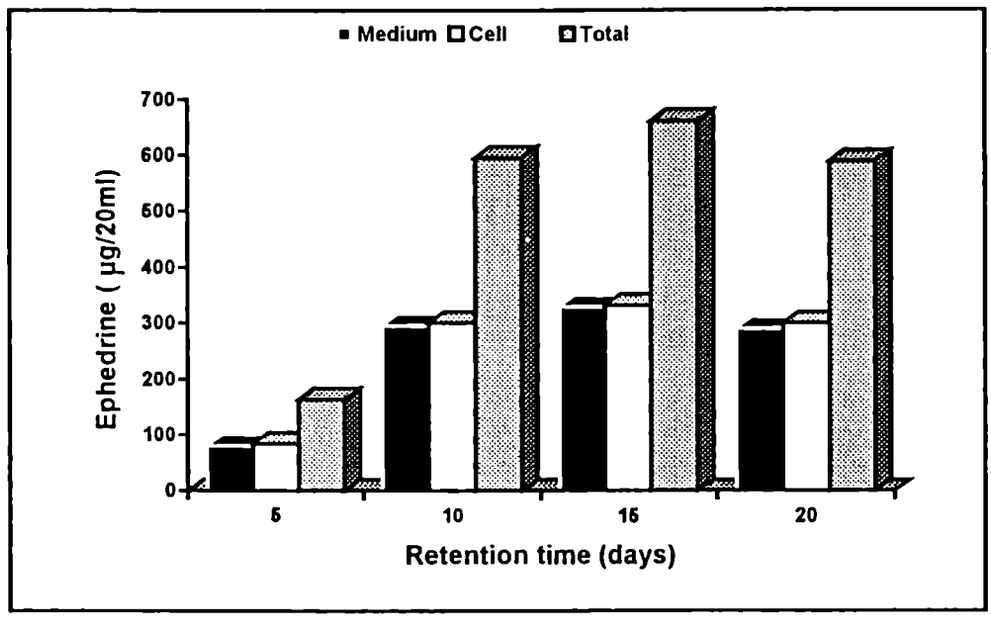
Results presented in Table 7.1 indicate that immobilization of viable cells (Plate 9) has effected about a two fold increase in total ephedrine production in *S.rhombifolia* Linn. compared to free cells

7.4 Discussion

The chemical potential of the normally slow growing plant cells (under batch condition) could be more efficiently exploited by immobilization. The use of immobilized cell beads is cost effective because of the possibility of reusing the "biocatalyst" and there is no need for the separation of the product from the catalyst.

Fig 7.5

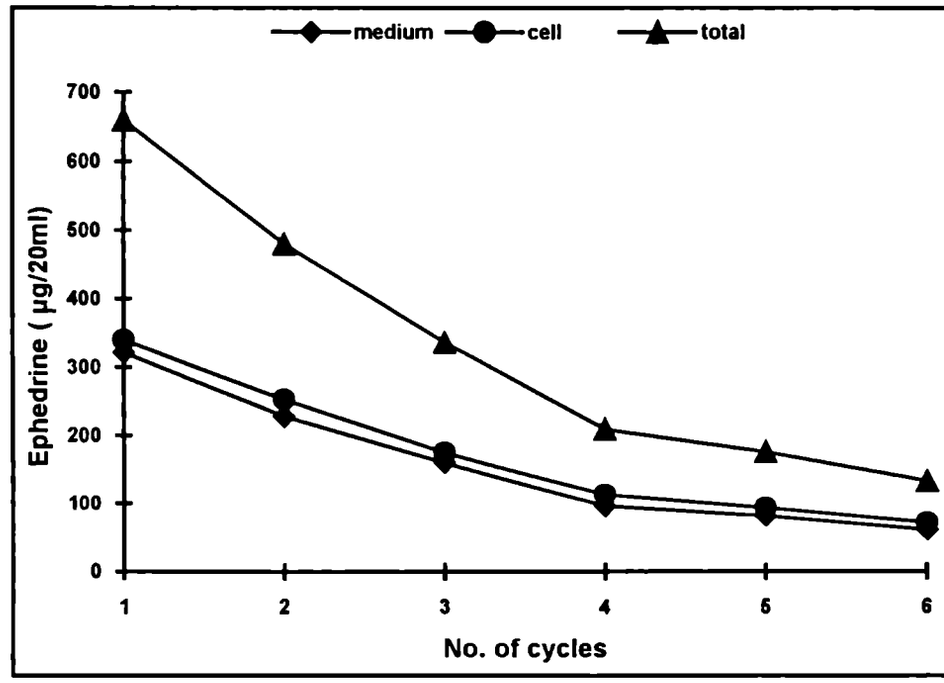
Optimisation of retention time of cell beads of *S.rhombifolia*.



1g cell was immobilized and incubated in 20ml medium for 10 days in an orbital shaker 90rpm at 25±2^{0}C under 1000 lux light intensity (16h phoptoperiod). The data reperesent average of 5 replications.

Fig 7.6

Optimisation of half life period of immobilized viable cell beads of *S.rhombifolia*



1g cell was immobilized and incubated in 20ml medium for 10 days in an orbital shaker 90rpm at 25±2^{0}C under 1000 lux light intensity (16h phoptoperiod).After each cycle the same beads were used to initiate the next cycle in fresh medium. The data represent average of 5 replications.

Table 7. 1

Impact of immobilization on ephedrine production

System	Ephedrine (μg/20 ml culture)		
	Cell	Medium	Total
Free cell system	170	126	296
Immobilized system	306	290	596

The data represent average of 5 replicates. 1g cells/1g cells immobilized in calcium alginate in 20 ml production medium was incubated in an orbital shaker (90 rpm) at 25±2°C under 1000 lux light intensity, for 14 days.

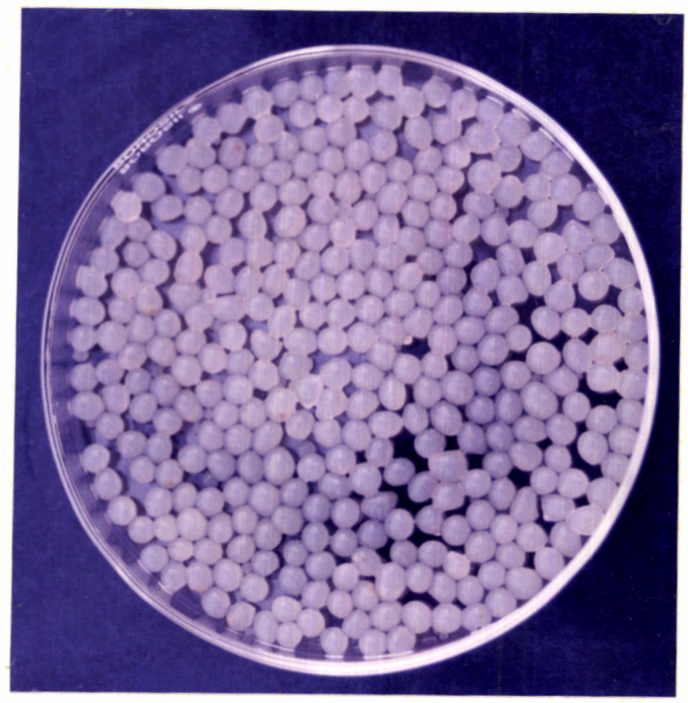


Plate 9 **Immobilized viable cell beads of *S.rhombifolia***

But the primary limitation of the immobilized plant cell culture is that the product must be extracellular. In the present system, only about 27% of the total alkaloid leaches out to the exterior under normal cell suspension culture system, whereas, when chitosan was used as a permeabilizing agent there was an enhanced rate of leaching (>50%) of the alkaloid.

Bordelius (1988) after a series of experiments concluded that immobilization of plant cells did not change the cell metabolism irreversibly. It was also shown that diffusion limitations were not so pronounced in slow growing immobilized plant cells (Hulst *et al.* 1985). In immobilized system, alginate could function as a glue between cells and thus mediate a cell to cell interaction, simulating that of a differentiated plant tissue. Of the various polymers used for entrapment of plant cells, only alginate could function in this manner and it was only in alginate entrapped cells where increased secondary product formation has been observed (Brodelius, 1985).

Since viability in calcium alginate beads, polyurethane foam pieces and freely suspended cells is comparable, calcium alginate was selected as the immobilizing matrix for the evaluation of the potential of the cells of *S.rhombifolia* to release ephedrine at enhanced level, in the present study. Another distinct advantage with this matrix is the reversibility of immobilization which enables investigation of the cells in free suspension subsequent to their entrapment, which is not possible with other matrices/supporting material.

In the present study, 1.5% of sodium alginate was found ideal for immobilization of *S.rhombifolia* Linn. cells (Fig.7.1). At this concentration, the beads were transparent which might have permitted light penetration inside the beads. As the production of ephedrine was influenced by light, the production was maximum for this concentration (540 µg/20 ml culture). At higher concentrations of sodium alginate, although the beads were very stable, the production was comparatively less. The same concentration was reported ideal for maximum capsaicin production in *Capsicum fruitisceus* (Johnson, 1993).

A cell concentration of 40% in the beads (Fig.7.2) was most ideal for maximum production of ephedrine (596 µg/20 ml culture). Plant cells being a slow growing system, unlike microbes, comparatively a higher concentration of cells in the beads were required for better production. Further, in the immobilization system, a production medium with limited major nutrients and plant growth regulators were provided in order to prevent the growth of the cells and the consequent bursting of the beads and leaching of cells in the medium which will upset the whole system.

The optimum curing time (Fig.7.3) of the immobilized cell beads of *S.rhombifolia* Linn. for maximum ephedrine production was 2 h (612 µg/20 ml culture). During the curing of beads, they shrink to a certain extent due to the exclusion of water, as the calcium ions diffuse into the beads and replace the sodium ions to form calcium alginate and bind the polymer chains more tightly together. This shrinking is different for different concentrations of alginate. If the complete

replacement of the sodium ions by calcium ions was not effected, the stability of the beads may not be retained. Further, as the plant cells are highly sensitive to stresses, the basal MS medium with 50 mM $CaCl_2.2H_2O$ was used for curing experiments instead of aqueous solution of $CaCl_2.2H_2O$ and beads were kept in an orbital shaker (90 rpm) under 1000 lux light intensity.

Optimal activation time (Fig.7.4) and retention time (Fig.7.5) for maximum ephedrine production were 18 h (636 µg/20 ml culture) and 10 (662 µg/20 ml culture) days respectively. The maximum level of production remained static up to 15 days.

Further, the studies on half life period of the immobilized cell beads of *S.rhombifolia* (Fig.7.6) suggest continuous use of immobilized viable cell bead for ephedrine production for 3 successive cycles of batch cultures.

CHAPTER VIII

Summary

8.1 *Sida rhombifolia* Linn. ssp. *retusa* (Linn.) Borssum, collected from the Aromatic and Medicinal Plants Division of Kerala Agricultural University, was used in the present study, for ephedrine production.

8.2 Fast growing friable calli were initiated from the leaf explants of *Sida rhombifolia* Linn. on Murashige and Skoog medium supplemented with 2.5 mg l^{-1} 2,4-dichlorophenoxy accetic acid and 1 mg l^{-1} benzyl aminopurine.

8.3 Initially the process parameters including temperature, light intensity, pH, carbon source, macronutrient combination, vitamin combination, amino acid supplement and plant growth regulators were optimized in callus cultures for maximum growth and ephedrine yield.

8.4 *S.rhombifolia* Linn. callus cultures recorded maximal growth and ephedrine yield at 25°C, pH 5.8 and under 1,000 lux light intensity.

8.5 Among the various major salt formulations tried, the MS major salt formulation promoted maximal growth and ephedrine production.

8.6 Among the carbon sources tested, sucrose promoted maximal growth and ephedrine production.

8.7 A combination of MS and B5 vitamin formulations favoured maximal biomass and ephedrine yield compared to individual additions.

8.8 Among the amino acids tested, the media supplemented with L-lysine and L-glutamine enhanced the biomass and ephedrine yield.

8.9 Among the various combinations of phytohormones tested, combination of 2.5 mg l^{-1} of 2,4-D and 1.0 mg l^{-1} of BAP resulted in maximal biomass and ephedrine production.

8.10 MS basal medium was modified (MSSR-4) and optimized by incorporating MS macronutrients and micronutrients, (MS+B_5) vitamin combinations, a phytohormonal combination of 2.5 mg l^{-1} of 2,4-D and 1.0 mg l^{-1} of BAP and supplemented with 1mM each of L-glutamine, glycine and L-lysine for maximal growth and ephedrine yield.

8.11 Growth analysis in callus culture indicated that the exponential growth phase is of 14 days after an initial 7 days of lag phase. This is followed by a progressive deceleration stage of 7 days before setting in of stationary phase of growth.

8.12 Ephedrine production was growth associated although maximum production was on 27^{th} day of callus culture.

8.13 Specific growth rate (μ) of 0.132/day and doubling time (td) of 5.25 days in callus cultures were observed during time course experiment.

8.14 Suspension culture was established from healthy friable callus.

8.15 Presence of *Bacillus* sp. as latent contaminant was observed.

8.16 From the antibiogram, ciprofloxacin and gentamycin were identified as the most sensitive antibiotics.

8.17 The contaminant bacteria could effectively be contolled by administering the minimal lethal concentration of gentamycin (14 μg ml^{-1}) and ciprofloxacin (18 μg ml^{-1}), to the growth medium, without adversely affecting the plant cell viability.

8.18 A pH of 5.0-5.5 was optimum for maximal biomass and ephedrine yield in suspension culture.

8.19 An inoculum concentration of 5% was optimum for maximal growth of culture.

8.20 Growth analysis in suspension culture revealed a typical sigmoid pattern with well defined lag (about 5 days), exponential (about 9 days) and stationary phases. The maximum growth was recorded on 14^{th} day of culture.

8.21 While ephedrine production started at the early logarithmic phase, it got accumulated more during the late logarithmic phase. However, maximum ephedrine production was noted on the 14^{th} day of culture.

8.22 The specific growth rate (μ) was 0.173 day^{-1} and the doubling time (td) was 4 days.

8.23 About 27% of the total ephedrine alone was released into the medium.

8.24 Repeated mutagenesis with both chemical (EMS & AO) and physical agents (UV) resulted in a mutant line which recorded a maximal enhanced ephedrine yield of 32%.

8.25 Among the various stress factors studied for their influence on ephedrine yield, phosphate stress was most effective for ephedrine production in suspension culture, and hence, phosphate was chosen as the limiting nutrient. In a two stage culture system, where the actively growing cells, on mid log phase (10^{th} day of culture), were transferred to a medium lacking the limiting nutrient, the ephedrine yield was enhanced by 2.6 fold.

8.26 When the actively growing cells were transferred to a medium with half the level of nitrate concentration, 46% increase of ephedrine yield could be effected. Similarly 29% increase in ephedrine yield was obtained by transferring the cells to sucrose deficient medium.

8.27 Addition of 0.75 mM L-phenylalanine promoted an increase of 1.78 fold ephedrine yield on 18th day of culture, whereas addition of 2 mM L-methionine resulted in 1.60 fold increase of ephedrine yield on 14th day of culture.

8.28 Addition of chitosan at 5 μg ml^{-1} resulted in 56.2% release of ephedrine, without affecting the cell viability percentage.

8.29 *S.rhombifolia* Linn. cell culture system was highly susceptible to fungal elicitor treatment and an increase of 2.65 fold ephedrine was recorded in elicitor treatment with 5% Mycelial Extract of *Pythium aphanidermatum* after a contact time of 4 days.

8.30 Mycelial Extract of *Rhizoctonia solanii* at 5% (v/v) level could enhance 2.21 fold increase in ephedrine production after a contact time of 4 days.

8.31 Among the various abiotic elicitors tried, calcium effected the maximum positive influence (1.76 fold increase) followed by manganese (1.52 fold increase) and zinc (1.36 fold increase).

8.32 Initially the process parameters that influence the preparation of immobilized viable cell beads were optimized. The concentration of sodium alginate, calcium chloride, cell concentration in the beads, curing time, activation and retention periods were optimized.

8.33 It was observed that 1.5% sodium alginate, and 2 h of curing time in half MS medium with 50 mM $CaCl_2.2H_2O$ favoured optimum gel stability and maximal ephedrine yield.

8.34 Immobilized cell beads with 40% cell concentration, yielded maximum ephedrine.

8.35 Activation period of 18 h and retention time of 10 days were recorded as optimal conditions for maximum ephedrine yield by immobilized cells.

8.36 Half life of the immobilized cells in shake flask cultures, after running 6 cycles of batch process was recorded as 3 cycles.

8.37 The NMR spectral data obtained for the isolated ephedrine correlated well with that of standard ephedrine.

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